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SYNTHETIC FRAGMENTS OF THE A CHAIN OF OVINE INSULIN

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SYNTHETIC FRAGMENTS OF THE A CHAIN OF OVINE INSULIN

The use of sulfur protective groups of differing acid lability

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SYNTHETIC FRAGMENTS OF THE A CHAIN OF OVINE INSULIN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
DE RECTOR MAGNIFICUS PROF.MR. F.J.F.M. DUYNSTEE,
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door

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Aan mijn ouders
Aan Marian,
Zazie en Brechje

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Many thanks are due to Dr. K.E.T. Kerling and the Laboratory of Organic Chemistry of the University of Leiden for the opportunity to use their peptide synthesizer.

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CONTENTS

C H A P T E R I

GENERAL INTRODUCTION

The insulin molecule consists of two polypeptide chains: an A and a B chain. The A chain contains 21, the B chain 30 amino acid residues. A characteristic feature of insulin is the presence of three disulfide bridges, a disulfide loop in the A chain and two disulfide bridges connecting the chains, resulting in a bicyclic system (figure 1).

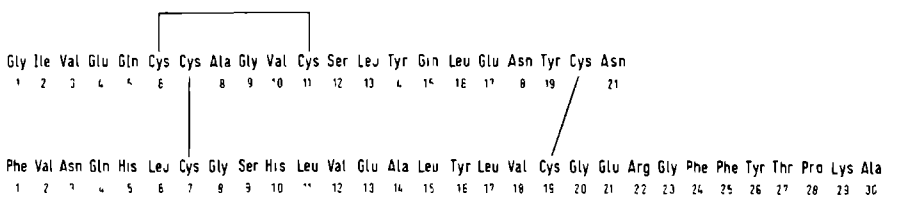

Figure 1. Structure of ovine insulin

In vitro syntheses of insulin have been undertaken quite actively by several research teams. Zahn[1] and Katsoyannis[2] as well as a Chinese group[3] have reported syntheses of insulin by random oxidation of the two synthetic chains at pH values ranging from 8-10.6. Insulin preparations, synthesized by these groups, have a biological activity in the order of 0.5-8%, although Katsoyannis has reported a preparation with higher activity. Admirable as these synthetic achievements may be, it should be noted that the activity of the preparations obtained, is of the same order of magnitude as the activity expected (3%) if

bridging by disulfide bonds occurs purely statistically during oxidation.

There are twelve ways in which the A- and B-chain can combine giving insulin isomers (figure 2); the possibility that more than one of these isomers may have insulin-like activity has to be envisaged.

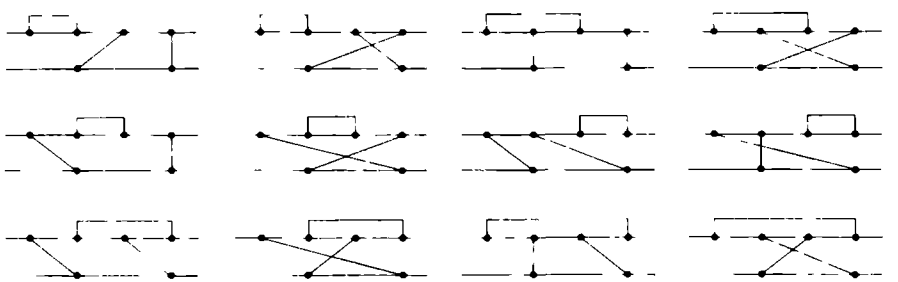

Figure 2. Insulin isomers

It seems quite apparent that the random oxidation procedure is not satisfactory, unless the desired disulfide arrangement is strongly favoured.

Some examples are known, in which such oxidations seem to proceed rather specifically. The reoxidation of completely reduced ribonuclease yielded 62% of a material, which had 80% of the original enzymatic activity[4]. If the oxidation was strictly random, only 1% activity should have been obtained. Therefore, the amino acid sequence of ribonuclease contains apparently information, necessary for the refolding of the amino acid chain into the correct secondary and tertiary structure.

The recent discovery of proinsulin gives an indication of the way in which insulin is prepared in biosynthesis. Proinsulin

is a one chain polypeptide in which the A and B chains of insulin are connected by a C chain (33 amino acid residues). Reduced proinsulin can also refold properly upon oxidation[13]; subsequent enzymatic cleavage of the C chain results in the formation of native insulin. Hence, one approach for a useful *in vitro* synthesis of insulin would be the preparation of proinsulin.

Another approach, directed towards the stepwise, specific introduction of disulfide bonds, was described by Zervas[5]. He used sulfur protecting groups of differing lability, in order to synthesize the protected A_{6-12} fragment with a disulfide bridge between A_6 and A_{11}.

A different approach to the formation of disulfide bonds, especially intended for the syntheses of multiple- and unsymmetrical disulfides, has been taken by Hiskey and co-workers. They were able to prepare unsymmetrical cystine peptides from S-trityl protected cysteine derivatives, using thiocyanogen as the oxidizing reagent[6]. This method allows selective bridging of two S-trityl thioethers in the presence of an S-benzhydryl thioether. Further investigations have shown that afterwards, under appropriate conditions, another disulfide bridge can be formed between two S-benzhydryl moieties, without disturbing the previously formed disulfide bond[7]. Using an insulin-like model system, Hiskey *et al* succeeded in this way in the synthesis of a tris-cystine peptide, composed of an "A chain" and a "B chain"[8]. This result demonstrates that thiocyanogen can be used to establish disulfide bonds in a selective and stepwise manner.

For the application of the thiocyanogen method in the total synthesis of insulin, a strategy for the preparation of the A

chain was developed which consists of a coupling of the S-trityl protected A_{14-21} fragment with an S-benzhydryl protected A_{1-13} fragment in which the correct disulfide loop (A_6-A_{11}) has already been formed. This disulfide bridge has to be formed from two S-trityl protected cysteine residues at A_6 and A_{11}, in the presence of the S-benzhydryl protection at A_7, by means of thiocyanogen.

During a stay in Hiskey's department we took part in the synthesis of the S-trityl protected A_{14-21} fragment in solution[9]. Prof. Hiskey agreed that we continued this work in Nijmegen with investigations on the solid phase synthesis of fragments of the insulin A chain, which should fit into his overall strategy. As is known, the solid phase method offers the possibility to synthesize peptides in a short time, and if a good reaction procedure is found, better reproducible results can be obtained by automatization of the procedure. We used two different resins in our investigation: a hydroxy-ethyl-sulfonyl-methyl resin[10] ("β-sulfon resin") and a resin bearing as functional group the *tert*-alkyloxycarbonyl hydrazide moiety[11] ("hydrazide resin"). From both resins a synthesized peptide can be released under conditions, mild enough to maintain desired protecting groups in side chains and at the N-terminal amino group.

An extensive study on the application of the β-sulfon resin in the synthesis of the A_{14-21} sequence is described in chapter II. The main problem is the introduction and cleavage of the C-terminal asparagine residue. The use of an amide protecting group (Mbh) in combination with other acid labile groups is described in detail as one of the possibilities to attack this problem.

Chapter III describes investigations on the use of the hy-

drazide resin for the synthesis of insulin fragments. The first part deals with methodological investigations. A method for a rapid and easy determination of amino acid incorporations is described. The attachment of asparagine and glutamine has received special attention. The investigations resulted in a good synthetic procedure for the A_{14-19} fragment, which was finally converted into the S-protected A_{14-21} sequence.

In chapter IV preliminary investigations on the synthesis of the remaining fragment A_{1-13} are described. They are restricted to procedures in solution because it was found (chapter III) that S-trityl protected cysteine derivatives are poorly incorporated during solid phase peptide synthesis.
As C-terminal protecting group a hydrazide-Boc function was chosen, which provides a possibility to couple the A_{1-13} sequence with the A_{14-21} fragment. Sulfur protecting groups were those needed to introduce selectively the desired disulfide loop. Although we succeeded in the synthesis of the fully protected tridecapeptide, it appeared impossible to introduce the disulfide bridge at the tridecapeptide level.
The introduction of the voluminous sulfur protected cysteine residues results generally in the formation of peptides which have inattractive solubility properties and are experimentally difficult to handle. Therefore it will be clear that the choice of the fragment in which the disulfide bridge will be formed is rather important. Our attempt to close the disulfide bridge in the fully protected A_{1-13} sequence remained therefore unsuccessful.
A priori, this disulfide loop can be introduced at various stages in the synthesis of the A_{1-13} fragment. Recently Hiskey published the synthesis of the A_{6-13} sequence, containing the correct disulfide bridge[12].

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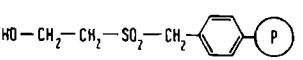
C H A P T E R II

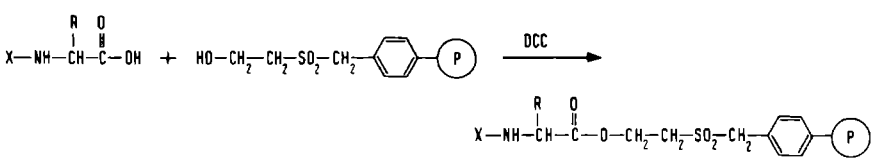
THE β-SULFON RESIN

II.1 INTRODUCTION

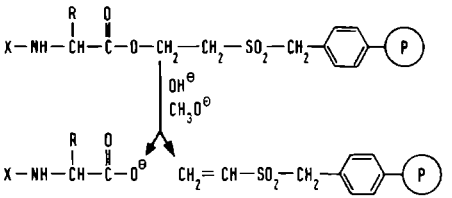
The β-sulfon resin, introduced by Tesser and Ellenbroek[1], offers the possibility that a peptide, synthesized on the resin, is cleaved from the polymer under alkaline conditions. Therefore it permits the use of acid labile sulfur and amino protecting groups.



In general the primary hydroxyl group is esterified with an N-protected amino acid by means of DCC:



Cleavage by β-elimination gives the free peptide within a few minutes:



This reaction is carried out in a dioxane–methanol mixture, containing enough sodium hydroxyde to give a concentration of 0.1 N. Some methyl ester is also obtained as a result of trans-esterification.

The main characteristics of this resin are:

-Its easy preparation from the chloromethylated Merrifield polymer.

-The possibility of direct esterification; this avoids the formation of quaternary ammonium salts, which are formed in the esterification of a chloromethylated polymer with the tri-ethylammonium salt of an N-protected amino acid.

-It permits the use of acid labile protecting groups of differing acid lability for sulfur and amino functions. If side chain protection with *tert*-butyl functions is used α-amino groups can be protected by more acid-labile groups, like Bmv or Bpoc.

-A protected peptide can be cleaved from the resin without loss of protecting groups.

-Peptides can be synthesized on a corresponding resin, containing a thioether function ($-CH_2-S-CH_2-$) instead of the sulfon group ($-CH_2-SO_2-CH_2-$).

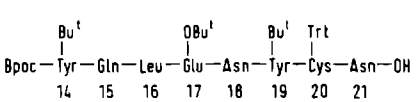
After completion of the synthesis, the sulfide can be oxidized to the sulfon and the peptide can be removed by β-elimination. The use of such a support is however limited by the oxidation

conditions required and would exclude the use of sulfur containing amino acids.

-Esterification of the hydroxyl function of this resin is carried out by means of DCC or N,N'-carbonyldiimidazole. These reagents cannot be used in the syntheses of peptides containing C-terminal asparagine or glutamine; this is a distinct disadvantage in comparison with the chloromethylated resin.

-Although the conditions of the alkaline cleavage are rather mild, even in comparison with those used in conventional alkaline hydrolyses of protected peptide esters, the incidental formation of side products cannot be excluded completely, for instance hydrolysis of peptide bonds, ring closure in C-terminal asparagine or glutamine, and perhaps cleavage of some protective groups, or racemisation. These side reactions however are not to be expected unless special amino acyl groups or combinations are present in the molecule.

In view of the last two characteristics certain difficulties had to be expected in the application of the β-sulfon resin to the synthesis of the A_{14-21} fragment of insulin in a protected form:



First of all there is the esterification problem of asparagine. In this chapter we will describe extensive studies on the attachment of an N-protected asparagine to the resin.

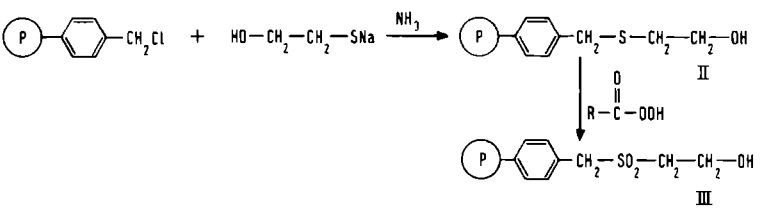
Another problem may arise upon cleavage of the peptide from the resin. The C-terminal asparagine residue can cyclize, which leads to the formation of side products. Furthermore, the pro-

tected cysteine residue might undergo partial β-elimination. From thin layer chromatography experiments however we concluded that an S-trityl containing model peptide, exposed to the basic conditions, used in the cleavage of the peptide from the resin, did not result in any detectable side product.

Additional investigations have been made with β-amide protected asparagine.

II.2 SYNTHESIS OF THE β-SULFON RESIN

Starting from the chloromethylated crosslinked polystyrene, this support could be easily prepared in a two step reaction, according to:



We could improve the oxidation of the sulfide to the sulfon resin by the use of 3-chloroperbenzoic acid; this peracid is easy to handle and its use does not result in the formation of coloured resins, as encountered in the oxidation reaction with pertrifluoroacetic acid. The oxidation with 3-chloroperbenzoic acid took only a few hours to come to completion, whereas the reaction with perbenzoic acid was only completed in 24 hours, due to the lower peracid content of the latter reagent[2].

II.3 DIRECT ESTERIFICATION OF BOC-ASN-OH TO THE β-SULFON RESIN

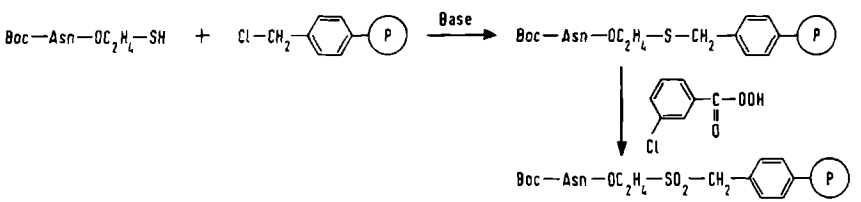
It is known that ring closure to a succinimide derivative or dehydration of the β-amide function can occur in reactions from N-protected asparagine in the presence of DCC[3]. Therefore the normal procedure for the esterification of an N-protected amino acid with the sulfon resin, using DCC as a condensing agent, cannot be applied.

An attempt to couple Boc-Asn-OH on the sulfon resin with DCC at $-15^{o}C$ in order to suppress the side reactions mentioned above, did not lead to any incorporation, as could be concluded from an I.R. spectrum of the resin. Esters of N-protected asparagine can also be prepared by the reaction of an alkyl chloride with the amino acid in the presence of dicyclohexyl amine as a base. Therefore we tried to substitute the hydroxyl function in the sulfide resin by chlorine. However, the reactions of the resin with thionyl chloride or phosphorus pentachloride in DMF or methylene chloride, did not result in the formation of the chloride, even at elevated temperatures.

Treitel[4] prepared esters from a tosylate and the sodium salt of an acid. The tosylates of the sulfide and sulfon resin could easily be prepared with tosyl chloride in pyridine. An I.R. spectrum clearly showed the absorptions due to the tosylate esters. However the reaction of the sodium salt of Boc-Asn-OH with the tosylate resins was not successful; as could be concluded from the I.R. spectra of the resins.

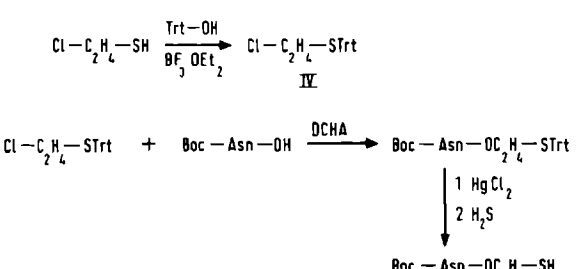
II.4 REACTIONS OF ASPARAGINE DERIVATIVES WITH THE CHLOROMETHYLATED RESIN

An alternative route for the synthesis of the Boc-Asn-sulfon resin involves the formation of an alkyl-sulfur benzyl-polymer bond from an asparagine derivative in which the ester bond has already been formed. Coupling of the 2-mercaptoethyl ester of Boc-Asn-OH with the chloromethyl polymer should afford the Boc-Asn-sulfide resin. Oxidation of the sulfide would lead to the desired Boc-Asn-sulfon resin (see scheme).



To this end a suitable sulfur protected 2-mercaptoethyl ester of the amino acid had to be prepared. The thiol protection has to be removed then, to realize substitution in the chloromethylated resin.

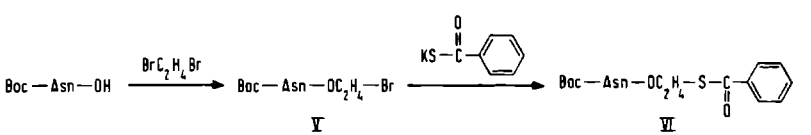
A useful sulfur protective function might be the triphenylmethyl group. It can be removed with mercury salts.



2-Chloroethyl triphenylmethyl sulfide (IV) was easily prepared from 2-chloroethanethiol. Quite unexpectedly however, no conversion could be observed in the reaction with an N-protected amino acid, under the conditions normally used in such esterifications.

Another possible sulfur protecting group is the benzoyl group. It is cleaved in dilute sodium methoxide solution within two minutes. The resulting thiolate salt can be used as such in the reaction with the chloromethyl resin.

The S-benzoyl-2-mercaptoethyl ester of Boc-Asn-OH was obtained in a two step synthesis by the preparation of the 2-bromoethyl ester of Boc-Asn-OH, followed by the substitution of bromine by the thiobenzoate ion:

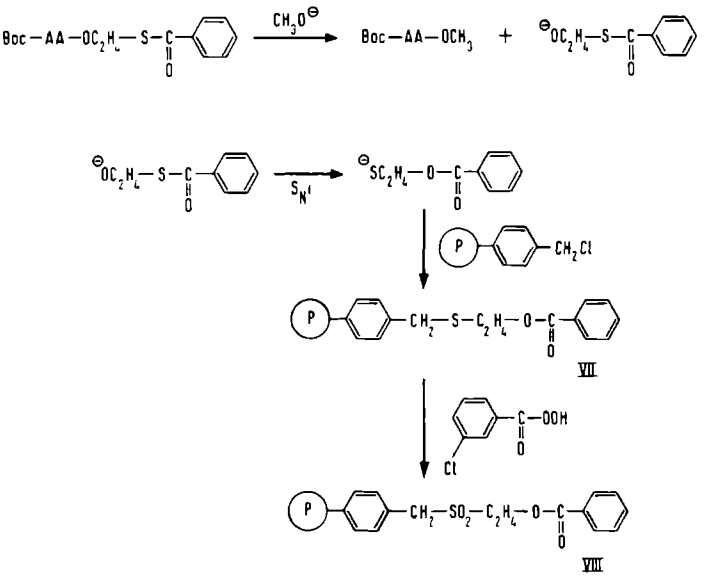


V VI

A substituted resin was obtained by treating this thiobenzoate derivative VI with one equivalent of sodium methoxide in methanol, followed by the addition of the chloromethyl resin in DMF. Its I.R. spectrum showed an ester carbonyl absorption at 1720 cm^{-1}. Combustion analysis showed the presence of 1.1% sulfur and a corresponding diminution of the chlorine content, compared with that of the starting polymer. Oxidation of this resin with 3-chloroperbenzoic acid resulted in the appearance of the sulfon absorption bands in the I.R. spectrum. Unfortunately acid hydrolysis of this resin showed the presence of only very small amounts of amino acid in the resin.

Similar results were obtained from a reaction of an appropriate glycine derivative with the chloromethylated resin.

From these facts and information gathered from thin layer chromatography we suppose that the results are caused by the following reactions:



It was shown that treatment of this resin (VIII) under basic conditions (in methanol-dioxane) results in the formation of benzoic acid and a trace of methyl benzoate. Our supposition is supported by the I.R. spectrum of the resin which appeared to be identical with that of a benzoate resin, obtained by treating the β-sulfon resin with benzoyl chloride.

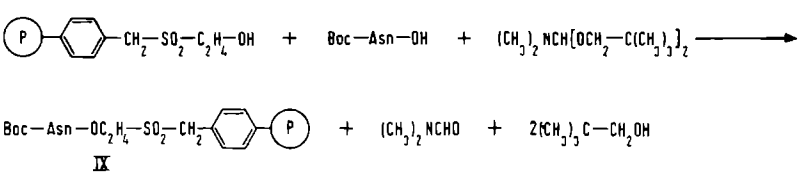
Our conclusion is that in VI transesterification is restricted to the O-ester, excluding cleavage of the S-ester

function. A second transesterification, probably intramolecular (favoured by a cyclic 5 ring intermediate), provides the thiolate ion, which reacts then with the chloromethyl resin.

II.5 ESTERIFICATION WITH N,N-DIMETHYLFORMAMIDE DINEOPENTYL ACETAL

A final attempt to incorporate an asparagine derivative into the β-sulfon resin was based on Schreiber's procedure[5] for the esterification of hydroxymethyl polystyrene with N-protected amino acids or peptides by means of N,N-dimethylformamide dineopentyl acetal.

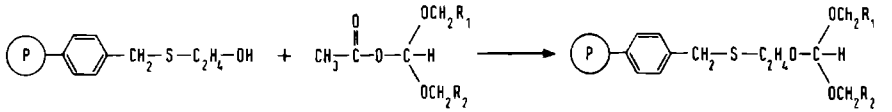
Adapted to our problem the method is formulated in the following scheme:



It appeared that the method could be used, indeed, for the esterification of Boc-Asn-OH with the sulfide resin as well as the sulfon resin.

A variation of this procedure, in which a mixed ortho ester of the β-sulfide resin was primarily formed and subsequently the ortho ester was brought together with an N-protected amino acid, appeared to be less successful.

Several ortho esters could be obtained in good yields, according to their I.R. spectra:



a $R_1 = R_2 = -CH_2OCH_3$

b $R_1 = R_2 = -CH_3$

c $R_1 = -H \quad R_2 = -CH_2CN$

Reactions of the ortho esters with an N-protected amino acid resulted in much lower amino acid incorporations as in the one step esterification using N,N-dimethylformamide dineopentyl acetal.

II.6 CLEAVAGE OF THE AMINO ACID FROM THE RESIN

It is known that alkaline hydrolyses of the esters from C-terminal asparaginyl peptides can be accompanied by side reactions[3]. However the complications do not always seem to be very serious. So, Katsoyannis[6] protected the carboxyl group of asparagine in position A_{21} of insulin during the synthesis of the A chain with a *p*-nitrobenzyl residue. After completion of the synthesis the ester bond was cleaved with sodium in liquid ammonia. Several authors[7,8] describe the saponification of insulin, completely esterified with methanol, without reporting side products.

We found however that treatment of the Boc-Asn-sulfon resin with a 2% 4N sodium hydroxyde solution in dioxane-methanol yielded a succinimide derivative as the main product.

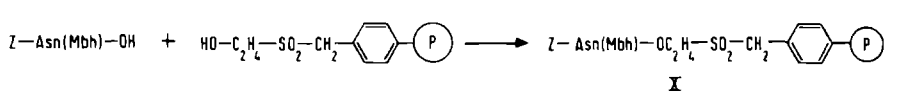
In chapter III.4.3 experiments will be described from which it can be concluded that in organic solvents, in the presence of dilute bases, succinimide formation takes place much faster than the β-elimination reaction, which was wanted.

II.7 REACTIONS WITH AMIDE PROTECTED ASPARAGINE

From the preceding sections it is clear that incorporation of N-protected asparagine in the sulfide or sulfon resin is possible (II.5), but that alkaline fission of asparagine from the sulfon resin leads mainly to cyclic products (II.6). Therefore we investigated the introduction of asparagine derivatives protected at the amine and amide residue. The presence of an amide protecting group permitting the use of DCC, would at the same time simplify the incorporation in the resin.

König and Geiger[9] recommend the 4,4'-dimethoxybenzhydryl moiety as a protective group for the side chain amide functions of asparagine and glutamine. It is easily introduced in asparagine with 4,4'-dimethoxybenzhydrol and an acidic catalyst like H_2SO_4, $BF_3.OEt_2$ etc., and can be cleaved with boron trifluoride etherate, which does not affect S-trityl functions. The stability towards base, induced by this protecting group, in glutamine derivatives seems to be rather certain, but sometimes hydrolysis of Asn(Mbh)-containing peptide methyl esters with 1N NaOH in dioxane-water resulted in the formation of side products.

Therefore we investigated the occurence of side reactions on a model system (X).



X

Z-Asn(Mbh)-OH was esterified on the resin with N,N-dimethylformamide dineopentyl acetal. The asparaginyl resin (X), thus

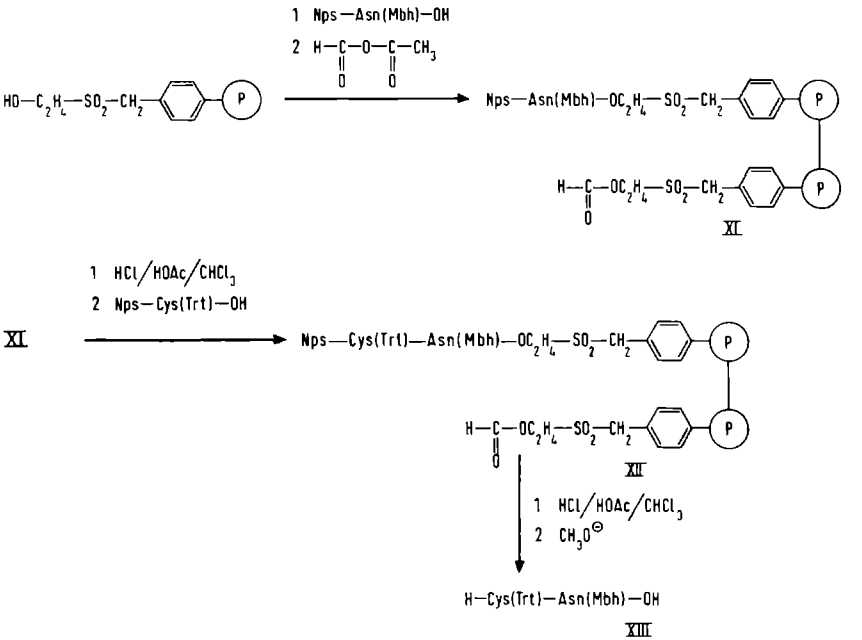
obtained, was treated with 0.04N NaOH in dioxane-methanol-water for two minutes. Analysis of the reaction mixture by means of tlc revealed the presence of minor side products, probably the methyl ester and the cyclic imide of Z-Asn(Mbh)-OH.

It was clear that the esterification rate of amide protected asparagine derivatives of this type into the β-sulfon resin with DCC was unfavourably influenced by the bulky substituent on the β-amide: rather low incorporations were obtained. Blocking of the unreacted hydroxyl groups, after the attachment of the amino acid to the resin was therefore necessary in the synthesis of larger peptides. This could be done by formylation with the formic acid-acetic anhydride reaction mixture[14].

The procedures described in this section for the introduction of asparagine derivatives into the β-sulfon resin and the cleavage of these esters without severe side reactions introduce a new difficulty, when peptides with C-terminal asparagine have to be prepared on the resin. The use of the Mbh-group might preclude the application of acid labile N^{α}-protecting groups like Nps, because their removal with methanolic HCl should also eliminate partly the Mbh group.

Therefore it was not surprising that the synthesis of the A_{20-21} sequence on the β-sulfon resin (see scheme), followed by cleavage of the dipeptide from the resin, resulted in the formation of at least five compounds, as was shown by thin layer chromatography.

Extensive purification of 110 mg of the reaction mixture resulted in a final yield of only 10 mg of a compound which was homogeneous on tlc and had the correct elemental analysis for dipeptide XIII.



II.8 CONCLUSION

Although the β-sulfon resin might have[10] distinct advantages over the chloromethyl resin, most frequently used in solid phase peptide chemistry, there are sometimes serious drawbacks.

They became very clear in our attempt to use this resin for the synthesis of the A_{14-21} sequence of insulin.

The attachment of asparagine to the β-sulfon resin proved to be troublesome. Only N,N-dimethylformamide dineopentyl

acetal as a condensing agent was found to be successful.

The alkaline cleavage of asparagine from the resin resulted in the formation of a cyclic imide.

The use of Mbh-amide protected asparagine diminished the amount of imide formed, but the compatibility with amino protective groups was too poor for application of this derivative in a synthesis of the A_{14-21} sequence by the solid phase method.

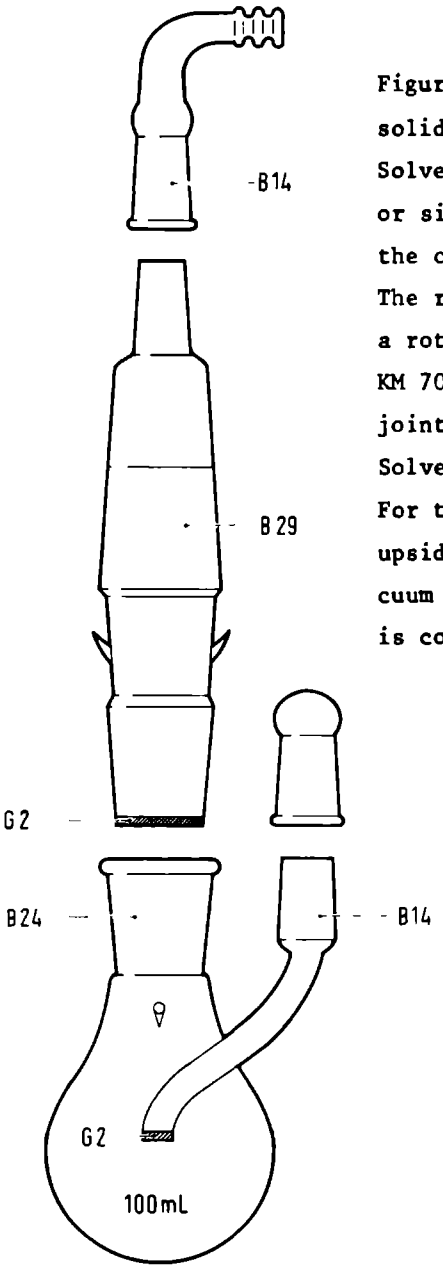


Figure 1. Reaction vessel for manual solid phase synthesis.
Solvents are added through the top- or side tube by applying suction at the other tube.
The resin suspension is agitated by a rotator (Ernst Keller, Basel, type KM 70/40) which fits the B29 ground joint.
Solvents are removed by suction.
For that purpose the vessel is turned upside down and put on a straight vacuum adapter (B29 ground joint), which is connected with a large reservoir.

II.9 EXPERIMENTAL

Melting points are uncorrected.

Optical rotations were determined with a Zeiss 366343 polarimeter.

Infrared spectra were taken on a Perkin-Elmer 257 infrared spectrophotometer with KBr pellets.

Thin layer chromatography (tlc) was conducted on precoated silica gel GF_{254} (Merck) plates. Spots were made visible by one or more of the following methods: a) U.V. fluorescence quenching, b) ninhydrin reagent, c) Reindel-Hoppe reagent.
A list of solvent systems used for tlc is given in the appendix.

Elemental analyses were carried out by the analytical department of the Laboratory of Organic Chemistry in Nijmegen. Analytical samples were dried *in vacuo* over P_2O_5 at 50-60°C.

For column chromatography, AG 1-X2 (chloride form, 200-400 mesh) was washed twice with 2N KOH, then with water until neutral; this was followed by two washings with 10% acetic acid and water until neutral. Before use columns were equilibrated with the appropriate solvent.

For manual solid phase peptide synthesis the reaction vessel shown in figure 1 was used.

Chloromethylated polystyrene (I) Styrene-2% divinylbenzene (200-400 mesh) beads were washed thoroughly with toluene, methylene chloride, 10% TFA in methylene chloride, 10% tributylamine in methylene chloride, DMF and ethanol to remove styrene and other low molecular weight products. Chloromethylation was performed according to Merrifield[11] by a reaction with chloromethyl methyl ether and $SnCl_4$ as a catalyst. Dependent on the resin used, reaction temperature was varied between 0° and 20° to obtain a useful percentage of chloromethylation (approximately 10% of the aromatic rings substituted; corresponding to about 3% Cl per g of resin).

β-sulfide resin (II) At -70°C ammonia (300-400 ml) was condensed in a three-neck roundbottom flask. After cooling 7.8 g of 2-mercaptoethanol were added dropwise under stirring, followed by the addition of 2.35 g of sodium, until the blue colour persisted. The solution was decolourized

by the addition of a few drops of 2-mercaptoethanol. Then 25 g of chloromethyl resin (0.65 mmole of Cl per g of resin) were added and the cooling bath removed. A slow stream of nitrogen was bubbled through the suspension until most of the ammonia had been evaporated. 300 ml of DMF were added and stirring was continued for 16 hours at 20°C. The resin was collected after the addition of 200 ml of methanol and washed with water, dioxane, acetic acid, methylene chloride and ethanol. The Beilstein test on Cl was negative. The I.R. spectrum showed strong hydroxyl absorption (see figure 2).

β-sulfon resin (III) A solution of 3.4 g of 3-chloroperbenzoic acid in 20 ml of methylene chloride was added in several portions to a suspension of 10 g of β-sulfide resin (II) in 50 ml of methylene chloride. On each

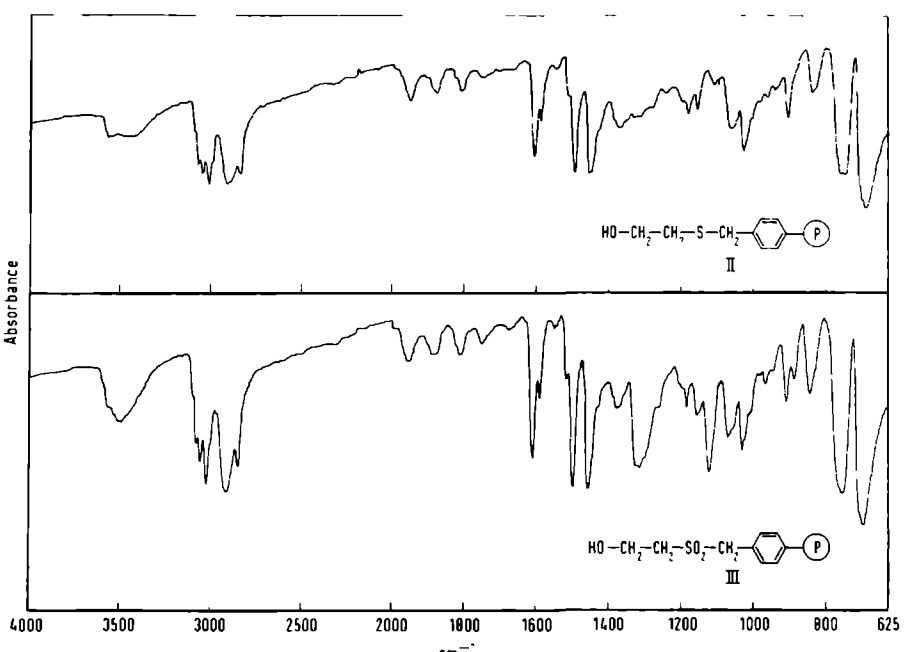


Figure 2. Infrared spectra of β-sulfide resin (II) and β-sulfon resin (III)

addition the temperature raised to the boiling point. The reaction mixture was then left at room temperature for 4 hours and the resin was collected and washed with methylene chloride and ethanol. An I.R. spectrum showed the characteristic sulfon absorptions at 1310 cm^{-1} and 1115 cm^{-1} (figure 2). From the sulfur content of this polymer it was calculated that this resin contained 0.60 mmole of the functional group per gram of resin.

2-Chloroethyl triphenylmethyl sulfide (IV) 2-Chloroethanethiol was prepared from 2-mercaptoethanol and conc. HCl[12]. 21.0 ml of boron trifluoride etherate were added to a suspension of 39.0 g of triphenylmethylcarbinol in 150 ml of acetic acid and 12.3 ml of 2-chloroethanethiol. The suspension was left at 80°C until a clear, slightly yellow solution was obtained (about 10 minutes). The thio ether crystallized as large plates upon cooling. Filtration and one recrystallization from acetic acid yielded 23.7 g (71%) of the pure product, mp 112-114°C.

Anal. Calcd for $C_{21}H_{19}SCl$: Cl, 10.46%. Found: Cl, 10.4%.

Boc-Asn-OC_2H_4Br (V) 5 ml of Et_3N (36 mmole) were added to a solution of 7.3 g of Boc-Asn-OH[13] (31.5 mmole) in 50 ml of DMF and 60 ml of 1,2-dibromoethane. After one hour a precipitate had been formed. The mixture was stirred at room temperature for 2 days and poured into 5% sodium bicarbonate solution. The oil which separated from the solution was extracted with chloroform, the extract washed with sodium bicarbonate solution, water, and dried over sodium sulfate. Then the solvent was evaporated. The resulting clear oil was freed from residual 1,2-dibromoethane by evaporation at 40°C *in vacuo*. Crystallization was effected from ether-diisopropyl ether-petroleum ether, yielding 8.12 g (76%) of the ester with mp 100-102°C, $[\alpha]_D^{22}$ -18.7° (c 1, MeOH), homogeneous (system A).

Anal. Calcd for $C_{11}H_{19}O_5N_2Br$: Br, 23.56. Found: Br, 23.5.

Boc-Asn-OC_2H_4-S-CO-C_6H_5 (VI) To a solution of 1.0 g of V (3 mmole) in 10 ml of DMF, 1.0 g of potassium thiobenzoate (5.8 mmole) was added. Precipitation of KBr started within a few minutes. After two hours the

mixture was poured into water. The precipitated ester was collected and washed with water. The dried product was recrystallized from methanol-ether-petroleum ether, yielding 1.12 g (96%) of white solid, mp 124-126°C, $[\alpha]_D^{22}$ -17.2 (c 1, MeOH), homogeneous (system A).

Anal. Calcd for $C_{18}H_{24}O_6N_2S$: C, 54.73; H, 6.10; N, 7.07; S, 8.09. Found: C, 54.7; H, 6.1; N, 7.0; S, 7.95.

Reaction of VI with the chloromethyl resin (VII) Into a solution of 2 g of VI (5.05 mmole) in 25 ml of DMF, 4 g of chloromethyl resin (5.95% Cl/g of resin) were suspended. The suspension was flushed with a stream of nitrogen gas, and 41 ml of 0.1232N $NaOCH_3$ in methanol (5.05 mmole) were added. The volume was reduced by evaporation and stirring was continued for two days. The resin was collected and washed with DMF, methylene chloride, dioxane, water, dioxane and ethanol. The chloride content of this resin was 5.28%. Sulfur content: 1.09%. An I.R. spectrum showed a broad ester vibration at 1720 cm^{-1}.

Substituted sulfon resin (VIII) To a stirred suspension of 3 g of resin VII in 20 ml of methylene chloride 0.5 g of 3-chloroperbenzoic acid, dissolved in 5 ml of methylene chloride was added. Stirring was continued for four hours. The resin was collected and washed with methylene chloride and ethanol. An I.R. spectrum of this resin showed sulfon absorptions at 1305 and 1115 cm^{-1}.

Action of base on substituted sulfon resin VIII To a mixture of 0.74 ml of dioxane, 0.25 ml of methanol and 0.01 ml of 4N NaOH a sample of resin VIII was added. After two minutes the resin was filtered and the filtrate acidified with 1 ml of acetic acid. Tlc of the filtrate showed the major component, liberated from the resin, to be benzoic acid.

Benzoyl sulfon resin A suspension of 50 mg of β-sulfon resin in 2 ml of methylene chloride was treated with 0.116 ml of benzoyl chloride (1 mmole) and 0.14 ml of triethylamine (1 mmole). The suspension was stirred for 15 minutes. The resin was collected and washed with DMF, methylene chloride, dioxane, water, dioxane and ethanol. A resin was obtained

which, according to its I.R. spectrum, was identical to resin VIII. Treatment with base, as described for resin VIII, delivered benzoic acid and a trace of methyl benzoate.

Boc-Asn-sulfon resin (IX) A suspension of 0.5 g of β-sulfon resin (III, 0.30 mmole) in 15 ml of benzene was treated with 640 mg of Boc-Asn-OH (2.75 mmole) and 0.77 ml of N,N-dimethylformamide dineopentyl acetal (2.75 mmole). The mixture was refluxed for three hours. The resin was collected and washed with DMF, methylene chloride and ethanol. Its I.R. spectrum showed that the amino acid had been incorporated quite well into the resin.

Action of base on resin IX A suspension of 50 mg of resin IX in the fission mixture* was stirred for one minute. The resin was collected and the filtrate acidified with acetic acid. Thin layer chromatography of the filtrate (system C) showed two spots. The major spot had an R_F-value, identical with that of the succinimide derivative, which was obtained from Boc-Asn-OMe by treatment with the fission mixture. The identification of the product is described in section III.4.3. The minor spot was shown to be Boc-Asn-OMe; this methyl ester was prepared on a microscale from Boc-Asn-OH and diazomethane, in dioxane as solvent.

Z-Asn(Mbh)-sulfon resin (X) β-sulfon resin (III) was treated with Z-Asn(Mbh)-OH[9] and N,N -dimethylformamide dineopentyl acetal as described for IX. An I.R. spectrum of this substituted resin showed characteristic absorptions at 1720 and 1660 cm^{-1}.

The resin was treated with the fission mixture as described for IX. Thin layer chromatography in systems B and C showed the product to be homogeneous and identical to Z-Asn(Mbh)-OH; in system D two small additional spots with higher R_F-values were found.

Nps-Asn(Mbh)-OH.DCHA The procedure of König and Geiger[9] for the preparation of the cyclohexylammonium salt was adapted for the synthesis of

* fission mixture: dioxane-methanol-4N NaOH = 7.5-2.4-0.1

the dicyclohexylammonium salt. The crude product was recrystallized from ethyl acetate and gave the pure compound in 80% yield. Mp 137-139°C $[\alpha]_D^{22}$ -22.3° (c 1, DMF), homogeneous (system C).

Anal. Calcd for $C_{37}H_{48}N_4O_7S$: C, 64.14; H, 6.98; N, 8.09. Found: C, 64.25; H, 7.1; N, 8.1.

Nps-Asn(Mbh)-sulfon resin (XI) From 5.2 g of Nps-Asn(Mbh)-OH.DCHA (7.5 mmole) the free acid was prepared by treatment of a suspension of the salt in ethyl acetate with citric acid solution at 0°C. The solution was dried and the solvent evaporated. The residue was dissolved in 15 ml of methylene chloride and shaken with 2.5 g of sulfon resin III for 10 minutes. 1.55 g of DCC (7.5 mmole) dissolved in 15 ml of methylene chloride were then added and the reaction mixture was left for 16 hr. The resin was washed with methylene chloride (3 times), DMF (3 times), and ethanol. An I.R. spectrum showed absorptions at 1720 and 1660 cm^{-1} of about the same intensity. Formylation of the unreacted hydroxyl groups was carried out in benzene, using an approximately tenfold excess of a formic acid/acetic anhydride reaction mixture[14], for 16 hr. The I.R. spectrum of this resin showed a much stronger absorption at 1720 cm^{-1}. From the sulfur content determined by elemental analysis it was calculated that this resin contained approximately 0.15-0.25 mmoles of amino acid per g of resin.

Nps-Cys(Trt)-Asn(Mbh)-sulfon resin (XII) A sample of 1.5 g of resin XI was treated as follows: it was washed three times with chloroform and once with 1.5 ml of a solution (1N) of HCl in acetic acid, diluted with 15 ml of chloroform. Then it was shaken during 10 minutes with the dilute solution just mentioned, next washed three times with methylene chloride and shaken for 10 minutes with a 10% Et_3N solution in methylene chloride and again washed three times with methylene chloride. After this pretreatment the resin was suspended into a solution of 516 mg of Nps-Cys (Trt)-OH[15] (1 mmole) in a mixture of 10 ml of methylene chloride and 3 ml of DMF, and left for 10 minutes. 206 mg of DCC (1 mmole) dissolved in 4 ml of methylene chloride were then added and the mixture was shaken for 2 hr. Finally the resin was washed three times with methylene chlo-

ride, three times with DMF and three times with ethanol.

H-Cys(Trt)-Asn(Mbh)-OH (XIII) In 1.5 g of the dipeptide resin XII the Nps group was removed by repeating the pre-treatment described in the preceding preparation. The resin was then washed three times with ethanol, dried and treated with the fission mixture for two minutes. The resin was filtrated and washed with acetic acid. The filtrate was evaporated, the residue dissolved in acetic acid, and water and sodium acetate were added to the solution. After 2 days at 4°C, a precipitate had been formed, which was collected and washed with ice-water; its weight was 110 mg. The crude product was precipitated twice from methanol-ether, yielding 30 mg of a product, that appeared to be inhomogeneous yet on tlc. This material was dissolved in 150 ml of a mixture of 1-butanol-methanol-water (1:1:1) and added to an AG 1-X2 * column (1.8 x 20 cm), which was eluted with 50 ml of the same solvent mixture, then with 1-butanol-methanol-0.03M acetic acid (200 ml) and next with 1-butanol-methanol-0.16M acetic acid (200 ml). Fractions of 5 ml each were collected. Absorbancy measurements at 254 mμ showed the presence of a main product and a minor compound in the fractions obtained with the second eluens used. Contents of the tubes containing the main product were pooled and concentrated to a small volume and the residue was lyophilized from 80% acetic acid giving 10 mg of a homogeneous product (system E).

Anal. Calcd for $C_{41}H_{41}N_3SO_6.2H_2O$: C, 66.56; H, 6.13; N, 5.68. Found: C, 66.7; H, 6.0; N, 5.5.

* AG 1-X2 is an anion exchanger manufactured by Bio-Rad Laboratories, Richmond, California, U.S.A.

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CHAPTER III

THE SYNTHESIS OF THE A_{14-21} SEQUENCE ON THE HYDRAZIDE RESIN

III.1 INTRODUCTION

In 1969 Merrifield and Wang introduced the *tert*-alkyloxycarbonyl hydrazide resin[1] in solid phase peptide synthesis. The desired peptide chain can be released from this resin with 50% trifluoroacetic acid in methylene chloride. In conventional solid phase peptide synthesis, with chloromethylated polystyrene as the solid support, acidic cleavage requires much stronger acids like HBr-TFA or anhydrous HF. The mild acidic conditions sufficient to cleave a peptide from the hydrazide resin, offer a possibility to isolate partly protected peptides, thus providing fragments suitable for further condensations. Especially in view of our strategy for the synthesis of fragments of the insulin A chain, an attractive feature of this resin is that treatment with 50% TFA-CH_2Cl_2 does not affect S-trityl groups.

Thus far, Merrifield published only two reports on peptide syntheses, in which the hydrazide resin was used. A protected tetrapeptide[1], and a protected decapeptide[2] were synthesized on this resin. For temporary α-amino protection the very acid sensitive Bpoc group was applied.

In this chapter some methodological investigations are described concerning this resin; an improved synthesis is given, and the application for the preparation of the protected A_{14-19}

fragment of insulin is described. This fragment could be coupled with the A_{20-21} dipeptide, resulting in the ultimate formation of the S-protected A_{14-21} sequence.

A comparison is made between syntheses performed by a manual method, using a reaction vessel as described in section II.9, and an automatized synthesis on a peptide synthesizer, following a corresponding procedure.

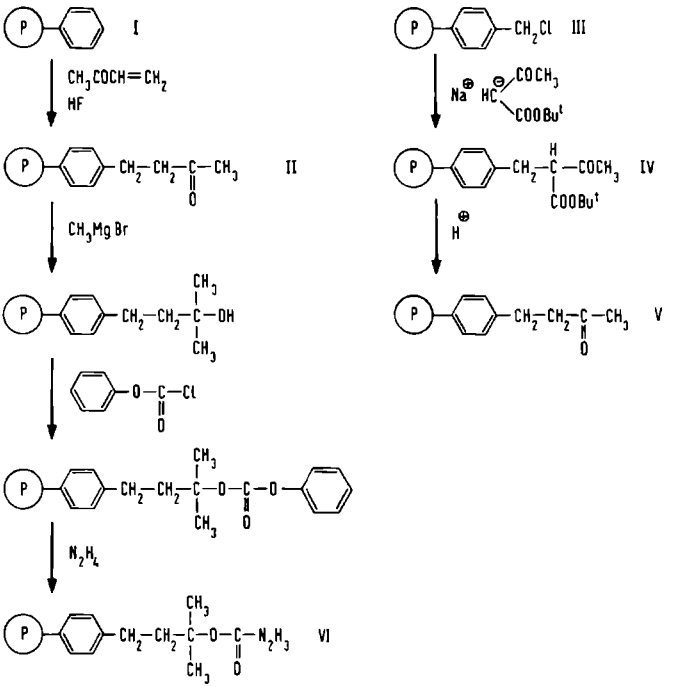
Section III.7 will deal with the synthesis of the starting compounds; an improved synthesis of tyrosine *tert*-butyl ether is given in this section.

III.2 SYNTHESIS OF THE HYDRAZIDE RESIN

Merrifield and Wang[1] described the synthesis of the *tert*-alkyloxycarbonyl hydrazide resin VI as presented in the left side of scheme 1. They used HF as catalyst in the Friedel Crafts reaction of copolystyrene-divinylbenzene (I) and vinyl methyl keton for the synthesis of the 3-oxobutyl resin II.

To avoid the use of the hazardous HF, we tried to synthesize the 3-oxobutyl resin II, using various other Friedel Crafts catalysts like $AlCl_3$, $BF_3.OEt_2$, H_2SO_4, $SnCl_4$ and BF_3 in H_3PO_4. It appeared from I.R. spectra that these catalysts could not be applied successfully in the synthesis of II. As a matter of fact, in our hands even the use of HF did not result in the formation of resin II. This may be due to the particular batch of polystyrene used.

In our search for another method to synthesize the 3-oxobutyl resin, we found that substitution of an acetoacetate carbanion in the chloromethyl resin presents a promising procedure to get II without the use of HF.



Scheme 1

The chloromethylated polystyrene resin III was treated with the sodium salt of *tert*-butyl acetoacetate. The β-keto acid ester resin IV was obtained being free from chlorine, and could well be characterized from its I.R. spectrum. Cleavage of the *tert*-butyl ester and decarboxylation were accomplished with 50% TFA-CH_2Cl_2 or by refluxing in 12N HCl-dioxane (1:1). The resulting 3-oxobutyl resin V had an I.R. spectrum identical to that published by Merrifield for the same resin obtained by the HF method.

The conversion of the 3-oxobutyl resin V into the *tert*-alkyloxycarbonyl hydrazide resin VI proceeded smoothly, as is described by Merrifield. It could be calculated from the nitrogen

content of the final product VI, that the over-all yield over five reaction steps was 71%.

We think this route has two major advantages:

1. The capacity of the hydrazide resin VI may be controlled by the extent of chloromethylation in the synthesis of the parent compound III.
2. The use of HF can be avoided.

III.3 METHODOLOGICAL INVESTIGATIONS

III.3.1 QUANTITATIVE MEASUREMENTS OF THE AMINO ACID INCORPORATION

The hydrazide resin can only be used in combination with N^{α}-amino protecting groups, which are more acid labile than the anchoring bond of the peptide to the resin. Useful examples are the Bpoc group, introduced by Sieber and Iselin[3], and the Bmv group, used by Southard in s.p.p.s.[4]. Both offer the possibility to measure the incorporation of an amino acid in the resin. When the Bpoc group is cleaved with 0.5% TFA-CH_2Cl_2, 2-(p-biphenyl)-propene is formed almost exclusively. The amount of this Bpoc cleavage product can be determined quantitatively by U.V.

A sample of the dried resin (4-7 mg) is weighed exactly and suspended in 50 ml of a 0.5% TFA solution in methylene chloride. After 10 minutes the optical density is measured at 274 mμ. From the molecular extinction in this solvent (ε=20.400) the incorporation can be calculated according to:

$$\text{mmole Bpoc-/g of substituted resin} = \frac{2.45\ d}{x}$$

in which: d=optical density; x=mg of resin

Bpoc determinations were carried out in duplo or triplo. The variations in the values obtained never exceeded 2-3%.
The resin particles were then collected and treated in a test-tube with 50% TFA-CH_2Cl_2 to cleave the peptide from the resin. The solvent was evaporated and a small calculated amount of DMF was added to reach a 1% concentration of peptide. This solution was spotted on tlc-, and sometimes on thin layer electrophoresis plates for a qualitative analysis of the reaction mixture.

For quantitative measurements of the incorporation of Bmv-protected amino acids, the amount of benzoylacetone, liberated from Bmv containing peptides, was determined by an analogous method; 3-8 mg of resin was weighed exactly and suspended in 25 ml of 0.4N HCl (aq.)-THF solution*. After 30 minutes the optical density at 307 mμ was measured and the incorporation calculated according to:

$$\text{mmole Bmv-/g of substituted resin} = \frac{1.67\ d}{x}$$

This formula is based on a measured molecular extinction of 15.000 for benzoylacetone in the solvent used.

To determine the yield for each individual coupling step the incorporation into the substituted resin (x) has to be converted into an incorporation value, related to the unsubstituted resin (c). General formulas for these conversions are:

*obtained by diluting 1 ml 6N HCl to 15 ml with THF

$$\frac{x}{1-x.M.10^{-3}} = c \quad \text{or} \quad x = \frac{c}{1+c.M.10^{-3}}$$

in which: x=incorporation expressed in mmole/g of subst.resin
c=incorporation expressed in mmole/g of unsubst.resin
M=molecular weight of the newly introduced residue

For a determination of free amino groups, left after a coupling step, Esko's method was applied[5]; 2-hydroxy-1-naphtaldehyde was allowed to react with a sample of the resin. The Schiff base obtained was cleaved with benzylamine and the optical density of the adduct was measured as described by Esko. This method could not be used for a determination of hydrazide groups on the resin.

It is clear that these quantitative measurements of amino acid incorporations are of the utmost importance to find optimal conditions for the individual coupling steps, and to become aware of possible irregularities in the synthesis of longer sequences. Additional information obtained from tlc and electrophoresis can offer an opportunity to get insight into the occurrence and nature of possible side reactions in the various coupling steps.

III.3.2 FORMYLATION OF FREE AMINO GROUPS

Before α-amino groups are liberated for the introduction of a new amino acid residue into the growing peptide chain, blocking of free amino groups which did not react in the previous coupling step, may be necessary to prevent the formation of failure sequences. The need for such a blocking was clearly

shown by Hagenmeyer[6]: At a hexapeptide level, $(Leu\text{-}Ala)_3$ bound to a polymer, there remained 21% of free amino groups after three repetitive couplings with Boc-Ala-OH. After one acylation with acetic acid anhydride, this value was lowered to 2%. At the nonapeptide level, however, acetylation gave only a small decrease in free amino groups.

Many other acylating agents have been described[7,8]. 3-Nitrophtalic anhydride leaves a free carboxyl function after reaction with the amino group which provides a possibility to separate incomplete sequences by ion exchange chromatography in the final isolation procedure[9].

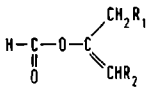
Acidic, or acid forming blocking agents, however, cannot be used in combination with acid labile groups like Bpoc or Bmv.

We found that the use of isopropenyl formate[10,11] offers a possibility to formylate free amino groups selectively, under neutral conditions and in a short time. The formylation of H-Tyr-OMe in CH_2Cl_2, using 10% excess of the reagent, resulted in 100% conversion into HCO-Tyr-OMe within 15 minutes.

VIIa VIII

NMR measurements indicated that phenol was formylated for less than 2% even after 10 days; methanol gave no more than 30% of methyl formate during this long reaction time.

We investigated the usefulness of two other analogous compounds (VIIb and c).



VII b $R_1 = H$, $R_2 = CH_3$

VII c $R_1 = OCH_3$, $R_2 = H$

Compound VIIb reacted considerably slower with tyrosine methyl ester (100% conversion after 120 minutes), whereas VIIc reacted slightly faster (100% conversion within 5 minutes) than unsubstituted isopropenyl formate.

Hydrazides in solution or hydrazides bound to a resin are also formylated: Boc-N_2H_3 and the hydrazide resin were converted into their corresponding formyl derivatives. The formylating agent did not react with Bpoc and Bmv functions: quantitative measurements of the Bpoc or Bmv content of a resin gave identical values before and after prolonged formylation. Acidic conditions used for Bpoc deprotection (0.5% TFA) or 50% TFA, used in the peptide releasing step, did not result in deformylation. The I.R. spectra, obtained before and after treatment of the formylhydrazide resin with 0.5% TFA in methylene chloride, were identical. Treatment of this resin for 30 minutes with 0.4N HCl (aq.)-THF, used for Bmv removal gave the same result. However, treatment of HCO-Tyr-OMe with 0.4N HCl (aq.)-THF resulted in approximately 20% cleavage of the formyl group after 18 hr.

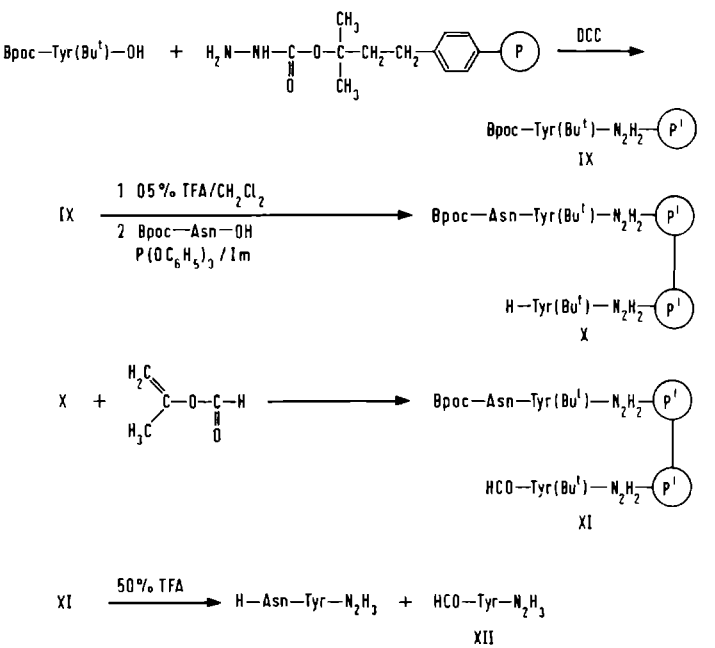
We conclude that isopropenyl formate or its methoxy derivative (VIIc) is suitable to be used as a terminating agent in s.p.p.s. because:

1. its low steric requirement makes rather complete formylation possible at resin sites, which are only with difficulty accessible.
2. it has a high reactivity and is selective towards amino groups; it does not affect peptide bonds or other functional

groups in the peptide synthesized.

3. it terminates by formylation, and the formyl-amino bond is stable under the reaction conditions used in our synthetic procedure.

An example of its application in s.p.p.s. is given in scheme 2.



Scheme 2

Resin IX, obtained from Bpoc-Tyr(Bu^t)-OH and the hydrazide resin in the usual way contained after Bpoc deprotection 0.38 mmole-NH_2 per gram of tyrosine *tert*-butyl ether substituted

resin. Coupling of Bpoc-Asn-OH with the triphenyl phosphite/ imidazole method, was not quantitative; resin X contained 0.07 mmole (18%) of free amino groups per gram of substituted resin. This value dropped to 0.015 mmole (4%) free amino groups after formylation with isopropenyl formate, resulting in resin XI. Investigation of the products obtained after a 50% TFA treatment showed the presence of a compound, which was identified as HCO-Tyr-N_2H_3 (by comparison with an authentic sample).

III.3.3 THE 2-BENZOYL-1-METHYLVINYL (BMV) FUNCTION AS AMINO PROTECTING GROUP

The Bmv group has been used in combination with a benzhydryl resin[4]. The N^{α}-protective group was removed under mild acidic conditions with 0.4N HCl (aq.)-THF within 30 minutes. In our synthesis this amino protecting group had to be used for N-protection of the cysteine residues, because Bpoc derivatives of S-trityl cysteine and S-benzhydryl cysteine could not be synthesized.

To combine this protecting group with the hydrazide resin, an investigation was made to establish the stability of the anchoring bond of the peptide to the resin. Because this anchoring bond closely resembles a Boc protection, we investigated the stability of the Boc group in a Boc-Gly-benzyl resin upon treatment with 0.4N HCl (aq.)-THF. I.R. spectra made clear that even after prolonged treatment no Boc cleavage could be observed.

If formyl groups, introduced to block free amino groups, should not be completely stable towards 0.4N HCl (aq.)-THF (section III.3.2), their cleavage should mainly concern amino

groups which appeared not to be well accessible to amino acid derivatives.

From these preliminary results we conclude that the Bmv amino protection might be used in combination with the hydrazide resin.

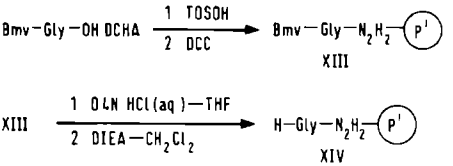
III.3.4 AZIDE COUPLING IN S.P.P.S.

The use of the hydrazide resin provides fragments suitable for further condensations. Since these fragments are obtained after detachment as hydrazides, an investigation of the possibility to couple hydrazides with a polymer-bound amino function by means of an azide coupling seemed worth-while.

Omenn and Anfinsen[12] applied the azide method to the coupling of a protected tripeptide azide with a polymer-bound pentapeptide. After cleavage from the support, about one-third of unreacted pentapeptide was found in the crude product. Visser and Kerling[13] linked an octapeptide by means of an azide coupling with a polymer-bound pentapeptide. In this case, only a few percent of unreacted pentapeptide could be detected in the crude material.

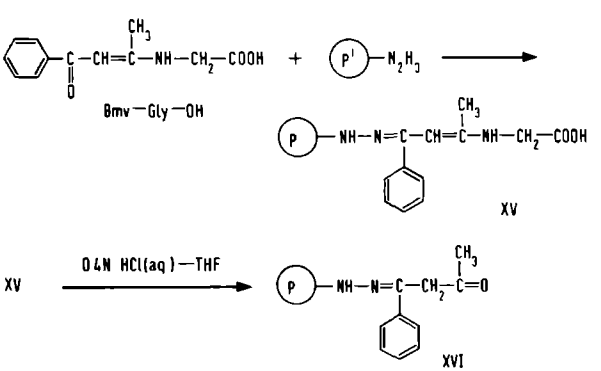
For our study we used a H-Gly-hydrazide resin and Z-Trp-Leu-N_2H_3, as the end-product Z-Trp-Leu-Gly-N_2H_3 was known from another project. The Esko method was applied for free amino group determination.

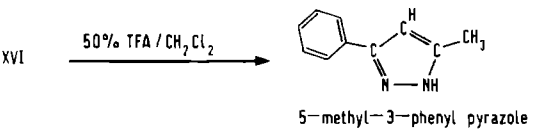
Bmv-Gly-OH.DCHA was coupled with the hydrazide resin as usual: anhydrous *p*-toluenesulfonic acid, dissolved in methylene chloride was added to a solution of the DCHA salt in the same solvent to obtain the free acid, and coupling was realized with DCC.



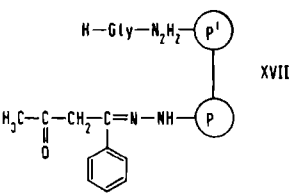
Free amino group determination of a sample of deprotected resin XIII revealed only 0.22 mmole free NH_2/g of resin, whereas 0.39 mmole/g would be found on quantitative incorporation. The introduction of the glycine derivative was therefore once more repeated with four equivalents of Bmv-Gly-OH. This raised the value of free NH_2/g of resin XIV only slightly to 0.23 mmoles.

Treatment of resin XIV with 50% TFA in methylene chloride and tlc of the resulting solution gave two spots. The spot with the lower R_F-value must have been H-Gly-N_2H_3; the spot with the higher R_F-value appeared to be the result of a side reaction which can be formulated as follows:

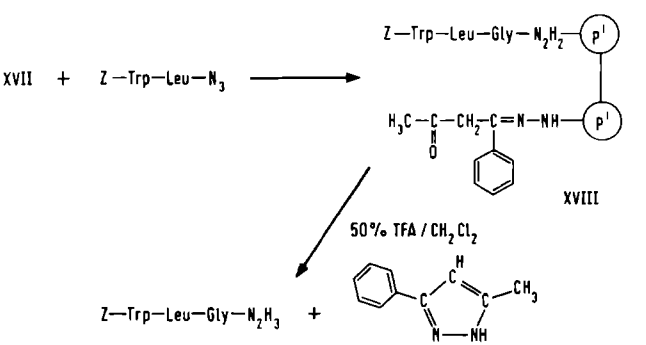




Treatment of resin XV with 0.4N HCl (aq.)-THF will liberate the amino acid, resulting in the formation of resin XVI, which, after cleavage with 50% TFA, gives 5-methyl-3-phenyl pyrazole. This compound had the same R_F-value as an authentic sample. Hence, resin XIV has to be written as:



For the azide coupling, resin XVII was treated with a fourfold excess of Z-Trp-Leu-N_3 (prepared, without isolation, from the corresponding hydrazide). As azide couplings in solution are



known to be rather slow, we decided to take samples after one, six and eighteen hours. Free amino group determination was carried out with the 1-hydroxy-2-naphtaldehyde method. After one hour a value of 0.003 mmole of free NH_2/g of resin was found, which corresponds to 1% unreacted amino groups. The same low value was found for the 6 and 18 hr samples. Therefore the azide coupling appeared to be extremely fast. Treatment of resin XVIII with 50% TFA resulted, again, in the formation of 2 spots on tlc; one was identical to the expected 5-methyl-3-phenyl pyrazole, the other appeared to be Z-Trp-Leu-Gly-N_2H_3.

It was therefore concluded that:

a. Bmv amino acids are not very suited to be condensed as the first amino acid with the hydrazide resin because their introduction results, after cleavage, in the formation of 5-methyl-3-phenyl pyrazole as a side product.
b. the azide coupling of Z-Trp-Leu-N_3 with resin-bound glycine is rather fast.

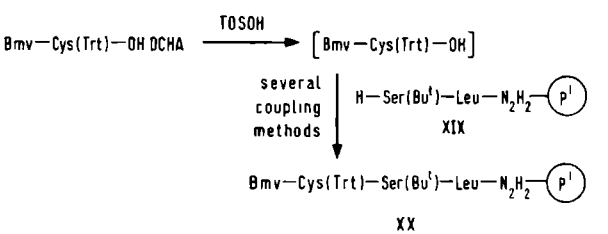
III.3.5 THE USE OF BMV-CYS(TRT)-OH IN SOLID PHASE SYNTHESIS

For a study of the applicability of Bmv-Cys(Trt)-OH in s.p.p.s. our experiences in the synthesis of the A_{6-13} sequence of ovine insulin are rather instructive. According to our strategy, this fragment holds two S-trityl cysteine residues and one S-benzhydryl cysteine residue. As stated before, the Bpoc group cannot be introduced into these compounds. Therefore, we prepared the Bmv derivatives of the S-protected cysteines. Contrary to the data reported by Southard[14], we found that Bmv-Cys(Bzh)-OH is crystalline, while its DCHA salt could

only be obtained as an oil (see III.7).

As starting compound for our study of the introduction of Bmv-Cys(Trt)-OH, the A_{12-13} dipeptide-bound hydrazide resin (XIX) was used which contained 0.39 mmole free NH_2/g of resin (0.36 mmole of Bpoc/g of resin before deprotection).

The necessity to use large volumes of 0.5% TFA in methylene chloride for deprotection of Bpoc derivatives was clearly shown in the synthesis of this A_{12-13} Ser-Leu-dipeptide resin. Bpoc-Leu-hydrazide resin was deprotected with a 100% excess of TFA. After coupling with Bpoc-Ser(Bu^t)-OH, Bpoc values of the obtained resin suggested quantitative incorporation. Tlc of a sample obtained after cleavage from the resin showed, however, 2 spots; amino acid analyses gave: Leu 1.0; Ser 0.25. This means that deblocking of the Bpoc group in the Bpoc-Leu-hydrazide resin had been incomplete. The use of a tenfold excess of TFA resulted in a dipeptide with a correct analysis: Leu 1.0; Ser 0.93.



The incorporation of the A_{11} residue, Bmv-Cys(Trt)-OH, was measured in dependence on the condensation procedure used. For each method the Bmv content of the resulting tripeptide resins XX are listed in table 1.

DCC, 90 min	0.107 mmole of Bmv/g of resin
DCC, 18 hr	0.165
DCC/HOBt*, 3 hr	0.275
DCC/HOBt*, 18 hr (repeated coupling)	0.280
N,N'-carbonyldiimidazole, 18 hr	0.05

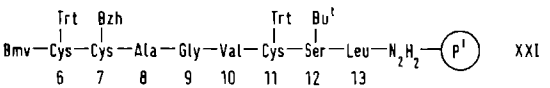
Table 1. Bmv content of tripeptide resin XX, obtained by various methods.

Apparently, the use of DCC alone results in rather bad incorporation; a reaction time of 18 hours instead of 90 minutes gives only partial improvement. The addition of HOBt gives better results, but even then the value obtained after 18 hours, corresponds with only 83% coupling.

After completion of the synthesis of the A_{6-13} sequence, using the optimal conditions just described for the introduction of further cysteine residues (A_7 and A_6), it appeared that the Bmv content of the octapeptide-bound resin XXI was only 43% of the Bpoc content of the parent A_{12-13} resin (both values based

*DCC/HOBt couplings of Bmv amino acids have to be carried out under nitrogen; otherwise yellow coloured resins are obtained.

on mmoles/g of the same resin). These data reveal that the



XXI

incorporation of Bmv-Cys(Trt)-OH in s.p.p.s. is far from quantitative. The application of this amino acid derivative in the s.p.p.s. of larger peptides will involve the necessity of troublesome purifications and, consequently, a rather low yield of the desired product. This could not fully be investigated in the peptide chosen for our study, because the protected nonapeptide hydrazide, obtained after deprotection of XXI, coupling with Z-Gly-OH and cleavage from the resin appeared to be completely insoluble.

III.3.6 COUPLING OF N-PROTECTED ASPARAGINE AND GLUTAMINE

The introduction of N-protected asparagine or glutamine is always carried out by the *p*-nitrophenyl ester method. Recent investigations[15] show that the *p*-nitrophenyl ester coupling in s.p.p.s. is notably slower than a DCC coupling; moreover, generally lower yields are recorded, even after prolonged reaction times or repeated couplings. Reaction of Boc-Val-ONp (threefold excess) with a H-Val-resin resulted, after 16 hours, in a 65% yield. Repeated coupling with three equivalents of Boc-Val-ONp for 16 hours raised the percentage to 80. Values between 90 and 98% were obtained with the corresponding DCC coupling[15].

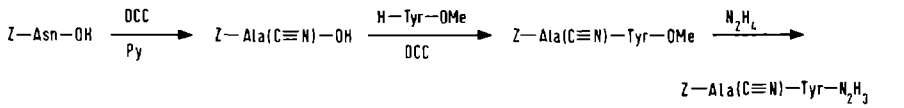
Mitin[16] published the triphenyl phosphite-imidazole method for the coupling of amino acids, and reported its application for glutamine and asparagine derivatives. We coupled one sample of the hydrazide resin with Bpoc-Tyr(Bu^t)-OH, according to his method. Another sample was treated with the same amino acid derivative in the presence of DCC. The Bpoc content of the two resulting resins showed that the coupling yield in the $P(OC_6H_5)_3$/Im method was 94% of that from the DCC condensation. Subsequent attachment of Bpoc-Asn-OH to the tyrosine resin with the $P(OC_6H_5)_3$/Im method resulted in only 82% incorporation.

It is not clear whether the low percentage must be ascribed to the coupling method only, or is a consequence of other factors as well.

König and Geiger[17] published the use of DCC/HOBt for the coupling of asparagine and glutamine derivatives in solution. Thus far, no reports have been published in which this elegant method has been applied in solid phase synthesis of asparagine or glutamine containing peptides. The coupling of Bpoc-Asn-OH with an H-Tyr(Bu^t)-resin using DCC/HOBt resulted in a dipeptide resin which contained 0.295 mmole Bpoc per gram of resin. This indicated 85% incorporation. No nitrile absorption could be detected in the I.R. spectrum. Without the addition of HOBt, a Bpoc content of 0.275 mmole was found. In this case the I.R. spectrum showed a very small absorption at 2260 cm^{-1}, due to the nitrile function.

The absence of nitrile formation in the former procedure was further established in the following way: Z-Asn-OH was coupled with a H-Tyr(Bu^t)-resin, using DCC/HOBt. The protected dipeptide hydrazide was released from the resin with 50%

TFA and investigated by tlc together with the corresponding β-cyanoalanine derivative, prepared for chromatographic purposes (see scheme). No detectable dehydration product could be observed in the dipeptide obtained from the resin.



III.4 N-TERMINAL AMINO PROTECTION IN S.P.P.S. WITH THE HYDRAZIDE RESIN

III.4.1 INTRODUCTION

The main characteristic of the hydrazide resin is that after completion of the synthesis, a hydrazide is released from the resin, which hydrazide can be used for coupling with other fragments. This means that the N-terminal amino group has to remain unaffected in the cleavage step.

Merrifield has used the benzyloxycarbonyl group for N-terminal amino protection. Unfortunately, that group cannot be removed selectively in the presence of an S-trityl function.

Among the known amino protecting groups which are stable towards 50% TFA in methylene chloride, and possibly can be removed selectively, we investigated the following:

1. The Cyoc group (cyano-*tert*-butyloxycarbonyl).
   This protective group is removed with weakly basic reagents *via* β-elimination. It was only recently introduced by Wünsch and Spangenberg[18].

2. The formyl group. The cleavage of N-formyl groups with hydrazine diacetate at 50°C was reported recently[19].
3. The Msc group (methylsulfonethyloxycarbonyl)[20].
   It resembles the Cyoc group; cleavage is *via* a β-elimination mechanism.

As model compounds for this investigation we chose the peptide derivatives XXII and XXIII.

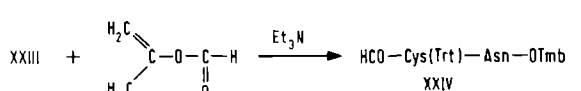
H—Cys(Trt)—Asn—OTmb
XXII

HCl H—Cys(Trt)—Asn—OTmb
XXIII

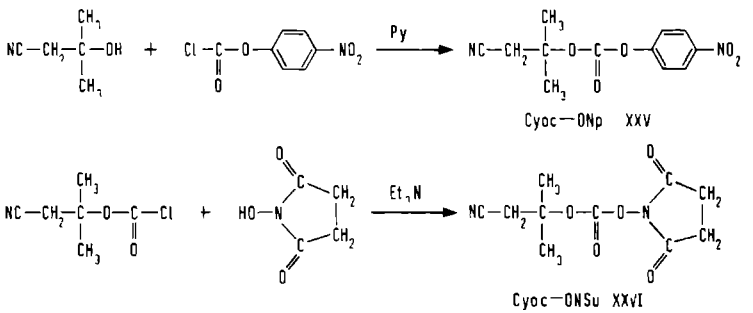
This choice was made because our investigations on s.p.p.s. with the hydrazide resin were intended for the synthesis of a protected A_{14-21} sequence in a form similar to that prepared previously in solution[21]. The latter derivative was protected at the C-terminal asparagine residue with a Tmb ester function. The model peptides chosen permitted investigation not only of the introduction of the above mentioned amino protecting groups, but also of their cleavage under circumstances which do not affect Tmb-ester and S-trityl functions.

III.4.2 THE INTRODUCTION OF TFA RESISTANT GROUPS

The formyl group was easily introduced on a micro scale into XXIII by means of isopropenyl formate VIIa or its methoxy derivative VIIc in the presence of an equivalent of Et_3N:



As reagents for the introduction of the Cyoc group we tried two appropriate carbonates:



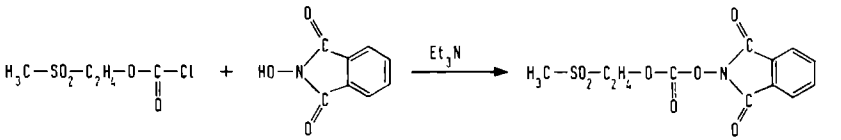
The corresponding phtalimidocarbonate could only be obtained as an oil.

The reactivity of the *p*-nitrophenylcarbonate XXV was rather low. Even with a large excess of XXV, the reaction with model peptide XXII was not complete after 5 hours.

The succinimidocarbonate XXVI reacted somewhat faster with the model peptide, but still too slow to be an attractive reagent. Therefore, the introduction of the Cyoc group was not very satisfying, and possible application of this protective group was not further investigated.

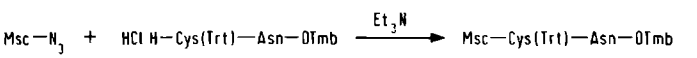
The Msc group, developed by Tesser[20], is usually introduced into amino acids and peptides by means of its *p*-nitrophenyl-carbonate. This carbonate, however, reacted rather slowly with our model peptide. The corresponding phtalimidocarbonate reacted faster; after 2 hours, complete conversion of our model peptide into the corresponding Msc-dipeptide was observed (tlc). The

phtalimidocarbonate XXVII was prepared according to:



Msc—OPht XXVII

The reaction between Msc-azide and the model peptide resulted after 4 days in the formation of a precipitate of the crystalline Msc-dipeptide:



XXVIII

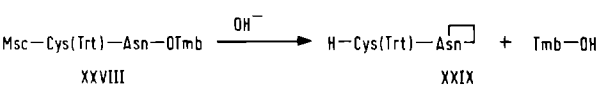
The obtained Msc-dipeptide XXVIII was used for studies on Msc cleavage conditions.

III.4.3 CLEAVAGE OF THE FORMYL- AND MSC GROUP

The formyl group can be cleaved by acid hydrolysis. As already pointed out in section III.3.2, 0.4N HCl (aq.)-THF hydrolyzes the formyl group only to a small extent. When formyl dipeptide XXIV was treated with this reagent at 40°C for 16 hours, indeed some formyl deprotection was observed, but large amounts of formyl dipeptide were still present and a side product had been formed as well.
It has been claimed[19] that cleavage of N-formyl groups by means of 2M hydrazine diacetate in methanol at 50°C for 20 hours is possible with maintenance of S-trityl and amide bonds.

However, when we treated H-Cys(Trt)-Asn-OH (section III.7) with hydrazine diacetate under the conditions cited above, tlc revealed that no trace of the original dipeptide was left. Therefore, we conclude that the formyl group cannot be selectively removed in the presence of an S-trityl protection.

Cleavage of the Msc group has been investigated in detail. Treatment of Msc dipeptide XXVIII with 10% excess of dilute sodium hydroxide solution for 15 seconds followed by acidification with acetic acid resulted in the formation of a product which was homogeneous on tlc and ninhydrin positive, but with a slightly lower R_F-value than the model peptide XXII.



XXVIII XXIX

Evaporation of the solvent *in vacuo* was accompanied by sublimation of 2,4,6-trimethylbenzyl alcohol, which was identified by its melting point. The residue could be identified by its I.R. spectrum as the succinimide derivative, XXIX.

Several other reagents were tested to investigate whether or not selective removal of the Msc group could be realized. Results of these experiments are summarized in table 2.

From these results we conclude that selective removal of the Msc group in peptides containing the -Asn-OTmb residue is rather problematic. Therefore, we changed our strategy for the synthesis of the A_{14-21} sequence. Instead of the Tmb ester of the S-protected octapeptide, the corresponding acid (with unprotected C-terminal asparagine) was prepared. This synthesis has been completed by coupling of an Msc hexapeptide hydrazide

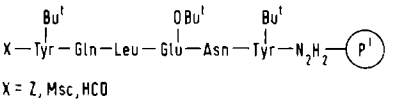
A_{14-19} (obtained from the hydrazide resin), with the S-protected dipeptide A_{20-21}, H-Cys(Trt)-Asn-OH, as will be described in the following sections.

	Msc cleavage	succinimide formation
Et_3N/DMF/H_2O (2:12:2) 18 hr 20°C and 6 hr 60°C	ca 50%	none
dioxane/MeOH/4N NaOH (7.5:2.4:0.05) 15 sec	100%	100%
dioxane/MeOH/4N NaOH (7.5:2.4:0.02) 5 min	100%	100%
N,N,N',N'-tetramethyl-1,8-naphthalenediamine ("proton sponge") 60 min	0%	0%
K_2CO_3/H_2O/DMF 5 min	0%	partly
TFA 60 min, 20°C	0%	Tmb ester cleavage

Table 2. Msc cleavage experiments on XXVIII

III.5 THE SOLID PHASE SYNTHESIS OF THE A_{14-19} SEQUENCE

Protected hexapeptide resins composed as presented in the following sequence were synthesized; various procedures were used.



The variations in the synthetic procedure concern mainly the incorporation of asparagine and of glutamine. The Mitin method ($P(OC_6H_5)_3$/Im) and the DCC/HOBt method were compared for the introduction of these residues. Sometimes the N-terminal dipeptide was introduced at the tetrapeptide level instead of further stepwise coupling. In this way, we avoided possible formation of a pyroglutamyl residue at the pentapeptide stage when the Bpoc group of the glutaminyl residue was eliminated.

For the manual syntheses (resulting in hexapeptide-bound resins XXX, XXXI and XXXII) a reaction vessel as depicted in section II.9 was used. The hexapeptide-bound resins XXXIIIa and XXXIIIb were synthesized in an automatized way; the same program was used as in the manual synthesis of XXXII. In general, all amino acid residues were introduced as Bpoc derivatives. Their incorporations were DCC mediated with the exceptions already mentioned.

With the exception of the first synthesis (product XXX), free amino groups were blocked after each coupling step with a formylating agent as discussed in section III.3.2. Moreover, in all five syntheses the incorporation of the amino acids subsequently introduced was measured as described in section III.3.1. The measured incorporation yields are represented in figure 1. The characteristics of each synthetic procedure are given in scheme 3.

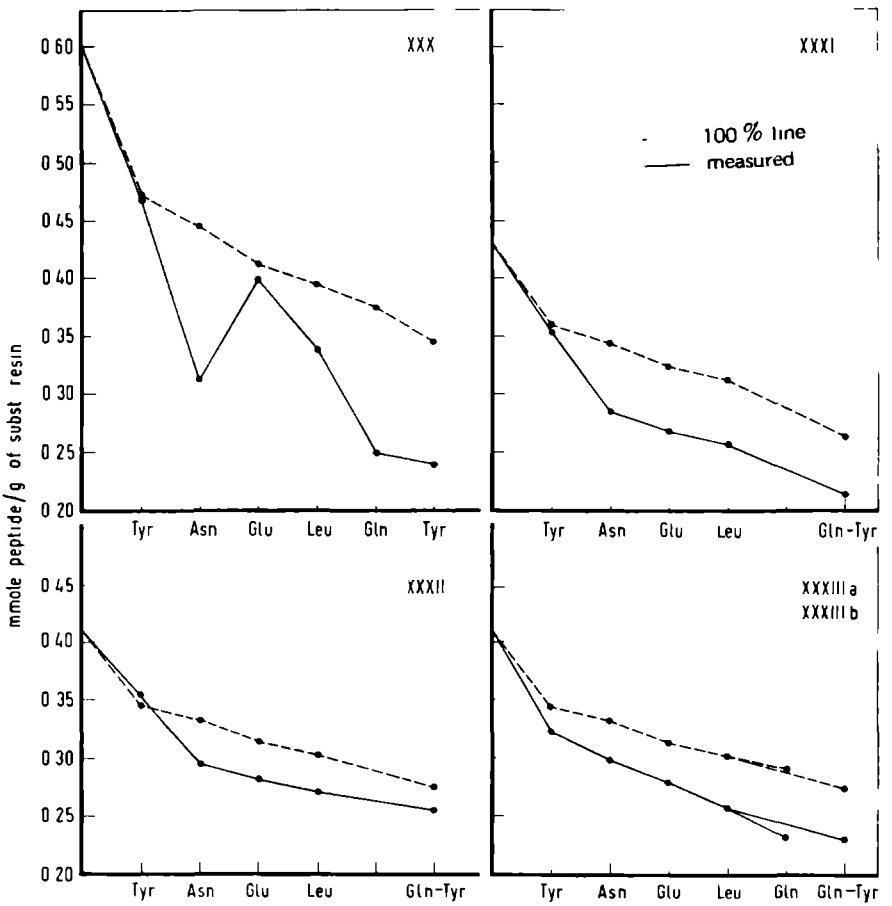


Figure 1. Bpoc contents of resins XXX-XXXIIIb

We carried out the synthesis of hexapeptide-bound resin XXX in a straightforward manner. The purity of intermediates was not established, but after each coupling, Bpoc measurements were carried out to determine the yield of incorporation. It is apparent from fig.1 that the incorporation of Asn and Gln were

resin*	procedure	formylation	coupling of -Asn- and -Gln-	residues introduced at the tetrapeptide stage	results	
XXX (0.60)	manual	no	$P(OC_6H_5)_3$/Im	1.Bpoc-Gln-OH 2.Bpoc-Tyr(Bu^t)-OH 3.Z-OPht	impure, even after extensive purification; low tyrosine content	
XXXI (0.43)	manual	yes	$P(OC_6H_5)_3$/Im	1.Bpoc-Tyr(Bu^t)-Gln-OH 2.Msc-OPht	impure A_{14-19}, resulting in 21% of pure N,S-protected A_{14-21}	
XXXII (0.41)	manual	yes	DCC/HOBt	1.Bpoc-Tyr(Bu^t)-Gln-OH 2.Msc-OPht and isopropenyl formate	52% of impure A_{14-19}, resulting in 53% of pure N,S-protected A_{14-21}	
XXXIIIa (0.41)	automatized	yes	DCC/HOBt	1.Bpoc-Tyr(Bu^t)-Gln-OH 2.Msc-OPht	60% of pure A_{14-19}	resulting in 58% of pure N,S-protected A_{14-21}
XXXIIIb (0.41	automatized	yes	DCC/HOBt	1.Bpoc-Gln-OH 2.Msc-Tyr(Bu^t)-OH	62% of pure A_{14-19}	

* The figures given in parentheses represent the capacity of the starting hydrazide resin

Scheme 3

not satisfactory at all. This must have led to failure sequences. In the end-product, the N-terminal Bpoc group was cleaved and replaced by the benzyloxycarbonyl group (with Z-OPht, see III.7). The liberated peptide was extensively purified (LH-20 gel filtration, counter-current distribution, preparative thin layer chromatography). However, it was not homogeneous on tlc. Amino acid analysis gave: Asp 0.96; Glu 1.97; Leu 1.0; Tyr 1.43. The low tyrosine content is to be expected when the already mentioned cyclization of the glutamine residue is involved in the synthesis.

At the end of the second synthesis (XXXXI) the Bpoc group was replaced by the Msc group with Msc-OPht (XXVII). It could be shown by I.R. that the introduction was rapid. The crude peptide obtained after cleavage from the resin was precipitated from DMF-ether. It appeared also to be impure on tlc, but it could be applied with success in the synthesis of the A_{14-21} sequence, as will be shown in the next section.

In the third, manually prepared hexapeptide resin (XXXII), the Bpoc group was changed partly by the formyl group and partly by the Msc function. After cleavage from the resin, the hexapeptide derivatives were precipitated once from DMF-ethanol-ether, yielding 52% of a product (based on Bpoc content at the hexapeptide level). Also these products were not completely pure on tlc. The formyl- and Msc peptide differ in solubility. In general, the former compound is less soluble in organic solvents. It dissolves well, however, in DMF/DMSO.

In the first automatized synthesis (XXXIIIa) the procedure

was quite the same as in the foregoing manual synthesis (XXXII). At the tetrapeptide level some of this resin was used for the synthesis of hexapeptide-bound resin XXXIIIb. Tlc at the pentapeptide level showed in addition to the ninhydrin positive pentapeptide hydrazide a minor spot which was ninhydrin negative and R.H. positive. This side product might have been the pyroglutamyl derivative.

The liberation and isolation of the Msc-hexapeptide hydrazides, obtained from the automatized synthesis XXXIIIa and b were performed simultaneously with that from XXXII.

Concluding remarks:

-DCC/HOBt coupling is far superior to the $P(O_6H_5)_3$/Im method for the incorporation of asparagine and glutamine.

-To obtain reasonably pure products, formylation of unreacted amino groups is necessary.

-The Msc group can be easily introduced at the hexapeptide level by means of Msc-OPht.

-If Bpoc-Gln-OH is incorporated into the tetrapeptide resin instead of the dipeptide derivative, Bpoc-Tyr(Bu^t)-Gln-OH, some pyroglutamyl derivative seems to be formed. It could not be detected, however, in the end-product.

-The purity of the hexapeptide can be rather well predicted qualitatively from Bpoc measurements performed during the synthesis.

-In the synthesis of XXXII and XXXIII the same batch of hydrazide resin was used. The attachment of the first amino acid was more than 10% lower in the automatized than in the manual synthesis (fig.1). This may be due to "aging" of the resin. The hydrazide resin used for the synthesis of XXXII

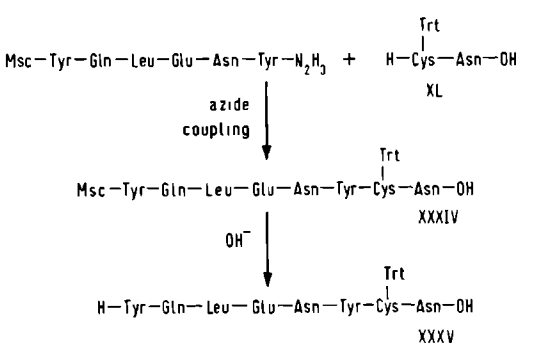
was freshly prepared; that applied to the synthesis of XXXIII was 6 months old.

On the other hand, incorporation of the second amino acid was rather low in the "fresh" resin, whereas in the aged one, the incorporation of the first and second amino acids were almost equal. It might be that aging of the resin is accompanied by complete closing of sites accessible only with difficulty in the fresh sample.

-Peptides obtained from automatized s.p.p.s. were perceptibly purer than peptides obtained from manually performed s.p.p.s.

III.6 THE SYNTHESIS OF THE S-PROTECTED A_{14-21} SEQUENCE

The N,S-protected octapeptide (XXXIV) was synthesized by an azide coupling between the A_{14-19} fragment (described in III.5) and the S-protected C-terminal dipeptide A_{20-21} (XL). Purification was done on an AG 1-X2 ion-exchange column, which proved to be a potent tool for the highly selective separation of peptides which differ in net negative charge[22]. The isolated octapeptide was treated with dilute base to liberate the N-terminal amino function.



Scheme 4

Also, the resulting S-protected octapeptide (XXXV) could be purified, if necessary, on an AG 1-X2 column.

According to scheme 4, the impure Msc-hexapeptide hydrazides obtained from resins XXXI and XXXII (manual synthesis) were converted separately into their azides and coupled with 25% excess of H-Cys(Trt)-Asn-OH (XL, section III.7). After 3 days at 4°C, the reaction mixture was diluted with 1-butanol-methanol-water (1:1:1) and applied to an AG 1-X2 column. Elution of the column with solvent mixtures containing stepwise increasing acetic acid concentrations released first the excess of dipeptide and other impurities. The octapeptide XXXIV was eluted by the solvent mixture 1-butanol-methanol-15% acetic acid. The acetic acid concentration needed was much higher than could be predicted on account of the net charge. Apparently the presence of the aromatic S-trityl function increased the amounts of acetic acid required for elution. This had the additional advantage that impurities which had about the same net charge as the octapeptide but which did not contain the S-trityl group, were eluted first.

The protected octapeptides were obtained in 21% yield (from XXXI) and 53% yield (from XXXII). According to tlc they contained only a trace of impurity, and they had the correct amino acid composition.

Msc cleavage of these A_{14-21} fragments was accomplished with dilute base in 1-butanol-methanol-water (1:1:1). After 5 minutes the solution was acidified with a few drops of $KHSO_4$ solution; then water was added. The resulting precipitate was applied to an AG 1-X2 column. The desired fragment was released in homogeneous form by the solvent mixture 1-butanol-methanol-1.5% acetic acid.

The same procedure as above was applied to the synthesis and purification of the N,S-protected octapeptide, starting with the hydrazides of resin XXXIII (automatized syntheses). The octapeptide (XXXIV) was obtained in 58% yield. It was homogeneous on tlc and gave the expected elemental analysis.
Deprotection of the N-terminal amino function was performed as described. The free octapeptide (XXXV) was obtained in 90.5% yield. No further purification was needed. It was homogeneous on tlc and it had a correct elemental analysis. Its amino acid composition was in agreement with the expected values.

Concluding remarks:

-Although the manual synthesis of the A_{14-19} fragments resulted in slightly impure peptide hydrazides, the A_{14-21} sequence could be obtained in pure form by ion-exchange chromatography.
-The N,S-protected and S-protected octapeptides, resulting from the automatized procedure, were obtained in higher yields than the same peptides obtained from the manual syntheses. Also, these compounds were very pure.

III.7 SYNTHESIS OF THE STARTING COMPOUNDS

One of the keystones of the investigation described in this chapter was the C-terminal dipeptide A_{20-21}. Several derivatives of this S-trityl dipeptide, listed in the following table, were prepared:

X—Cys(Trt)—Asn—Y

XXII	X = H	Y = Tmb	XXXVII	X = Nps	Y = Pmb
XXIII	X = HCl.H	Y = Tmb	XXXVIII	X = Boc	Y = Tmb
XXIV	X = HCO	Y = Tmb	XXXIX	X = Boc	Y = OH
XXVIII	X = Msc	Y = Tmb	XL	X = H	Y = OH
XXXVI	X = Nps	Y = Tmb			

With the exception of the Msc dipeptide (XXVIII) all of these derivatives appeared to be amorphous.

We showed that selective cleavage of the Nps group in the presence of acid labile S-trityl and Tmb or Pmb ester functions is possible (XXIII).

Boc-Cys(Trt)-Asn-OH (XXXIX) was prepared from the succinimide ester of Boc-Cys(Trt)-OH (not isolated) and asparagine. The N,S-protected dipeptide was obtained in 88% yield. Small impurities could be separated by ion-exchange chromatography on AG 1-X2. Removal of the Boc function of this dipeptide with $BF_3.OEt_2$ gave the S-protected dipeptide (XL) as a homogeneous product. Treatment of the N,S-protected dipeptide with anhydrous TFA gave, besides XL, a minor side product.

The syntheses of XXII and XXXVIII have already been described by the author elsewhere[21]. The preparation of the other derivatives mentioned in the table is described in the experimental part.

The procedure described by Schnabel[23] was used for the preparation of the various Bpoc amino acid derivatives needed. The stable reagent, [2-*p*-biphenyl-isopropyl]-*p*-biphenyl carbonate, was treated with a solution of the Triton B salt of the appropriate amino acid. The purity of the products was checked by tlc in two systems, by optical rotation measurements and by elemental analyses. We found that the CHA salt of Bpoc-Ser(Bu^t)-OH crystallizes much more easily than does the corresponding DCHA salt.

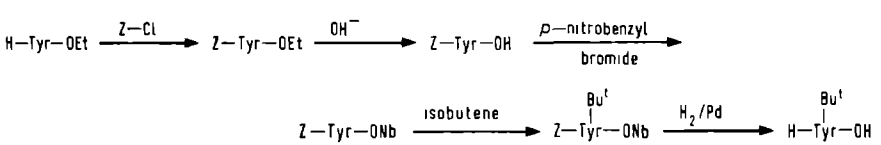
Bpoc-Tyr(Bu^t)-Gln-OH (XLI) was obtained in pure form in 78% yield from the reaction of glutamine with the N-hydroxysuccinimide ester of Bpoc-Tyr(Bu^t)-OH.

Several methods have been described for the recrystallization of Bpoc-Asn-OH[23,24]. We obtained the best results by the

addition of DMAC to a boiling suspension of Bpoc-Asn-OH in acetonitrile until a clear solution was obtained; cooling of the solution provides the N-protected asparagine. No trace of the free amino acid could be detected by tlc.

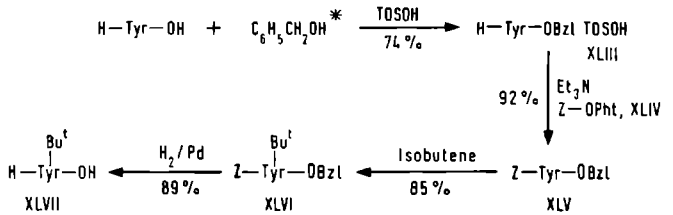
The elegant procedure described by Southard[14] for the direct preparation of the DCHA salts of Bmv amino acids by means of DCHA, benzoylacetone and the appropriate amino acid was easily applied to S-trityl cysteine and glycine. The same procedure applied to S-benzhydryl cysteine gave, however, an oil which did not yield a crystalline product in spite of all attempts. Treatment of an ethyl acetate solution of the oily DCHA salt with an aqueous citric acid solution gave a precipitate of the free acid (XLII). This could be recrystallized from methanol. No change in melting point was observed after storage at 4°C for a year.

As starting material for the recently published[21] preparation of Bpoc-Tyr(Bu^t)-OH.DCHA, we used H-Tyr(Bu^t)-OH, prepared according to procedures given by Wünsch[26] and Schröder[27]. The synthetic procedure reported by Wünsch is given in the following scheme:



Schröder described a direct *t*-butylation of Z-Tyr-OEt, followed by hydrolysis of the ethyl ester. The resulting compound, which was isolated as its DCHA salt (Z-Tyr(Bu^t)-OH.DCHA) can eventually be converted into tyrosine *t*-butyl ether. The key intermediate, Z-Tyr(Bu^t)-OEt, however, was obtained as an oil.

These time-consuming procedures could be simplified by using the benzyl ester of tyrosine. The benzyl ester derivatives crystallize easily and give high yields in all individual reaction steps, resulting in an over-all yield of 51.5% (see scheme):



*For the synthesis of H-Tyr-OBzl.TOSOH (XLIII) it is of crucial importance that the benzyl alcohol used be free of peroxides, which turn the reaction mixture black within a short time.

III.8 CONCLUSION

The main conclusion of the investigations described in this chapter is that the hydrazide resin has certain advantages over the conventional chloromethylated resin for the synthesis of peptide fragments which have to be used in the synthesis of longer sequences. The protected peptide hydrazides that it provides are useful starting compounds for fragment condensation. Unfortunately, peptide hydrazides do not have very attractive solubility characteristics, which can hamper the purification of the obtained fragments. This drawback may become a serious problem when cysteine residues with bulky apolar S-protecting groups are present in the peptide chain. The introduction of such cysteine derivatives in s.p.p.s. raises another

problem because their incorporation is rather low.
Because incomplete incorporation has been found also with other amino acids, measurement of the incorporation yield is necessary after each step in s.p.p.s. on a hydrazide resin. The use of Bpoc and Bmv protecting groups for α-amino functions make such measurements readily possible.

Our methodological investigations led to a scheme for the synthesis of the A_{14-19} fragment which could be applied successfully in either a manual or an automatized procedure.

The *p*-nitrophenyl ester couplings of asparagine and glutamine can be avoided by using DCC/HOBt for the introduction of these amino acids.

Products obtained from an automatized synthesis were pure and were obtained in high yields, whereas the manual synthesis of the A_{14-19} fragment resulted in peptides slightly contaminated with impurities.

In the extension of the A_{14-19} fragment with the A_{20-21} dipeptide, containing S-trityl cysteine and asparagine, the need for an N-terminal protecting group in the A_{14-19} sequence which can be cleaved under non-acidic conditions has clearly been shown. In view of the cleavage procedures for the Msc group, selected for this purpose, C-terminal asparagine must be used unprotected. The introduction of the Msc group by means of a carbonate in s.p.p.s. permitted the synthesis of the A_{14-21} sequence; its selective removal in the presence of an S-trityl function provided the S-protected A_{14-21} sequence in excellent yield and with high purity.

III.9 EXPERIMENTAL

General remarks given in section II.9 are also applicable to this section.

A list of solvent systems used for tlc is given in the appendix. Compounds containing tyrosine residues and/or hydrazide functions were also made visible with the Barton reagent ($K_3Fe(CN)_6/FeCl_3$).

Thin layer electrophoresis was carried out on cellulose coated plates at 400 V for 1 hr in a pyridine-acetate buffer (pH=4.6).

Acid hydrolyses of peptides for amino acid analyses were performed in constant boiling HCl at 110°C for 24 hr in evacuated tubes. Values have not been corrected for amino acid destruction.

Performic acid oxidations of cysteine containing peptides were carried out by oxidizing 1-2 mg of peptide with 1 ml of performic acid (9.5 ml of formic acid, 0.5 ml of 35% hydrogen peroxide) for 3 hr at 0°C, followed by evaporation of the solvent *in vacuo*.

Coupling and deprotection of each amino acid residue in s.p.p.s. was carried out according to schedules A and B.

For the automatized s.p.p.s., a peptide synthesizer from Schwarz Bio Research was used.

t-Butyl acetoacetate adduct of chloromethylated polystyrene (IV) *t*-Butyl acetoacetate (18.5 ml, 0.113 mole) was added to a freshly prepared solution of 2.6 g of sodium (0.113 mole) in 60 ml of ethanol. Ethanol was added up to a total volume of 100 ml, resulting in a 1.13 molar solution of the sodium salt. Chloromethylated polystyrene (20.0 g; 2.26% Cl) was added to 40 ml of the sodium salt solution and the ethanol evaporated *in vacuo*. 15 ml of DMF were added and the mixture was concentrated to a small volume *in vacuo*. Another 25 ml of DMF were added and the reaction was allowed to proceed with stirring for 2 hr at 70°C. The resin was filtered and washed with several portions of DMF, dioxane, dioxane-water, dioxane, methylene chloride and ethanol. An I.R. spectrum showed characteristic absorptions at 1735, 1715 cm^{-1} as well as 1255 cm^{-1}. The absence of chlorine in the resin was confirmed by a Beilstein test.

Schedule A. Coupling of Bpoc-amino acids, diimide mediated.
Designated for 1.5 g of resin (0.6-0.9 mmole).

step	reagent	vol(ml)	time(min)
1	Bpoc-AA-OH solution[1] (CH_2Cl_2 or CH_2Cl_2/DMF)	10	2
2	DCC/CH_2Cl_2[2)3)4)]	9	90[5)]
3,4,5	CH_2Cl_2 wash	25	5,2,2
6,7,8	DMF wash	25	5,2,2
9,10,11	EtOH wash	25	5,2,2[7)]
12,13,14	CH_2Cl_2 wash	25	5,2,2
15	10% formylating agent/CH_2Cl_2	18	60
16,17,18,19	CH_2Cl_2 wash	25	5,2,2,2[7)]

Schedule B. Cleavage of Bpoc group.

step	reagent	vol(ml)	time(min)
1	0.5% TFA/CH_2Cl_2[6)]	44	2
2	0.5% TFA/CH_2Cl_2	44	10
3,4,5	CH_2Cl_2 wash	22	2,2,2
6	10% DIEA/CH_2Cl_2	25	2
7,8,9	CH_2Cl_2 wash	25	2,2,2
10,11,12	EtOH wash	25	2,2,2
13,14,15	CH_2Cl_2 wash	25	2,2,2
16	10% DIEA/CH_2Cl_2	25	10
17,18,19,20	CH_2Cl_2 wash	25	2,2,2,2[7)]

[1)] The Bpoc amino acid solution was prepared just before use. Acids were liberated from their DCHA salts by means of a 2N $KHSO_4$ solution at 0°C.

[2)] Four equivalents of DCC and amino acid per mmole of resin were used.

[3)] When DCC/HOBt couplings were applied, one equivalent of HOBt per amino

acid, dissolved in DMF, was added to the amino acid solution. Two additional DMF washings (2 minutes each) were added after step 2 to prevent clogging of the filter.

4) In case of $P(OC_6H_5)_3$/Im couplings, 1.5 equivalent of $P(OC_6H_5)_3$ and imidazole per amino acid were added. Couplings were carried out in pure DMF at 40°C for 18 hr. Before and after the coupling, three additional DMF washings (2 minutes each) were added.

5) Couplings of Bmv amino acids were continued for 16 hr; DCC/HOBt couplings of Bmv derivatives were conducted in a nitrogen atmosphere.

6) For Bmv deprotection, step 1 and 2 of schedule B were changed into a 25 ml of 0.4N HCl(aq.)-THF washing, followed by a 30 minutes cleavage step with 25 ml of the same solvent system. Before and after this deprotection step two THF washings (2 minutes each) were applied.

7) Measurements on resin samples were performed after three EtOH washings and drying *in vacuo*.

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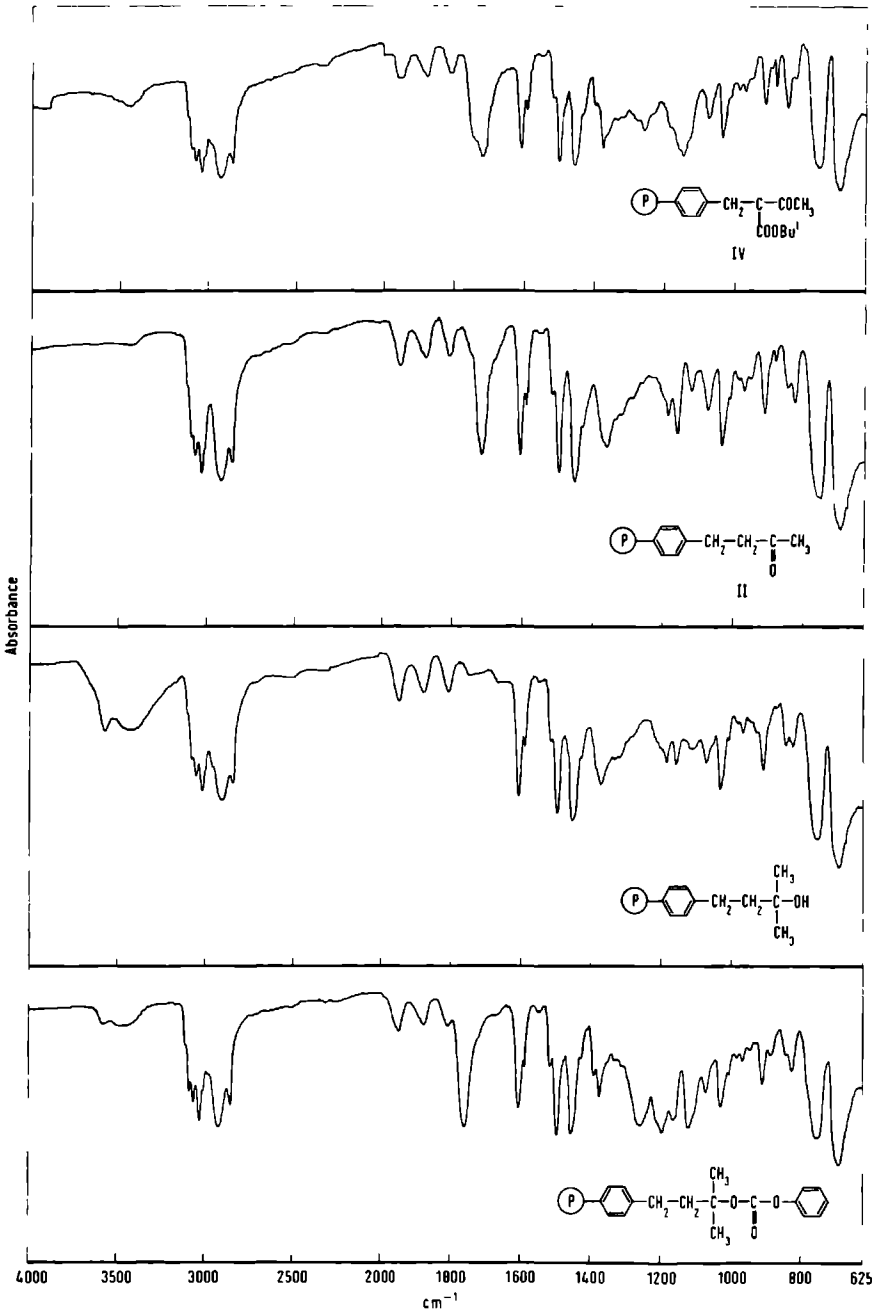
3-Oxobutyl resin (V) The β-keto acid ester resin (IV, 20 g) was suspended in 50 ml of 50% TFA in methylene chloride and the suspension rotated for 1 hr. The resin was filtered and washed thoroughly with methylene chloride, 10% DIEA in methylene chloride, methylene chloride and ethanol. The I.R. spectrum of this resin showed an absorption at 1715 cm^{-1} and the absence of absorptions at 1735 and 1255 cm^{-1}.

t-Alkyloxycarbonyl hydrazide resin (VI) Starting from the 3-oxobutyl resin V, the hydrazide resin was prepared according to Merrifield and Wang[1]. The 2.26% chlorine containing polymer resulted in a hydrazide resin which contained 1.21% nitrogen (VIb, 0.43 mmole hydrazide per g of resin). The capacities of two other batches were 0.6 mmole (VIa) and 0.41 mmole (VIc), respectively. The over-all yield in these three experiments was 71%, based on the chlorine content of the chloromethylated resin. I.R. spectra of the intermediate resins are given in figure 2.


Absorbance
IV
II
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
625
cm⁻¹

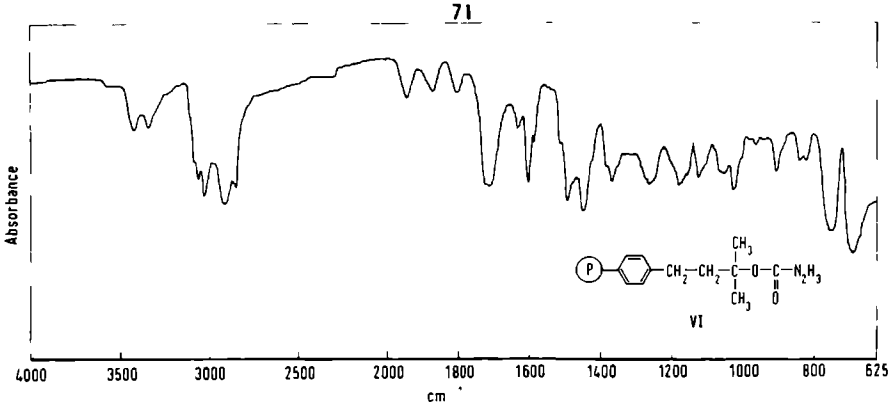


Figure 2. Infrared spectra of *t*-alkyloxycarbonyl hydrazide resin (VI) and its intermediates.

Isopropenyl formate (VIIa) The synthesis of this reagent and the other formylating agents (VIIb, VIIc) has been described[10,11] or will be described elsewhere[27]. They were usually obtained contaminated with the corresponding ketone. For the formylation of the hydrazide resin and other hydrazides, pure isopropenyl formate was used.

HCO-Tyr-OMe (VIII) H-Tyr-OMe (195 mg, 1 mmole) was suspended in 1 ml of methylene chloride. After the addition of 0.2 ml of a 50% solution of isopropenyl formate in acetone (10% excess), a clear solution was obtained. After 15 min, tlc showed the absence of starting material. A precipitate was formed after 60 min which, after the addition of ether, was collected and dried yielding 212 mg (95%) of VIII, mp 144-145°C, $[\alpha]_D^{22}$ +38.4° (c 1, MeOH), homogeneous (system C).

Anal. Calcd for $C_{11}H_{13}NO_4$: C, 59.19; H, 5.87; N, 6.27. Found: C, 59.1; H, 5.9; N, 6.25.

Bpoc-Tyr(Bu^t)-hydrazide resin (IX) Bpoc-Tyr(Bu^t)-OH was liberated from its DCHA salt by means of a $KHSO_4$ solution, and coupled with 1 g of hydrazide resin VIb, containing 0.43 mmole of hydrazide functions, according to the general schedule A. Liberated 2-(p-biphenyl)-propene, a free amino group determination, as well as its weight increase in-

dicated a quantitative incorporation. The product obtained after cleavage from a resin sample was homogeneous as shown by thin layer electrophoresis and was identical to an authentic sample of H-Tyr-N_2H_3 (obtained from H-Tyr-OCH_3 upon treatment with $N_2H_4.H_2O$ in methanol), mp 199-201°C.

Bpoc-Asn-Tyr(Bu^t)-hydrazide resin (X) Schedule B for deprotection of resin IX, followed by schedule A (step 1 till 11) for a $P(OC_6H_5)_3$/Im coupling was applied. The Bpoc content of this resin as well as a free amino group determination revealed an 82% yield for the asparagine incorporation. Electrophoresis of the liberated peptide showed a major and a minor component.

Formylated Bpoc-Asn-Tyr(Bu^t)-hydrazide resin (XI) The formylation steps (15-19) in schedule A were applied to dipeptide resin X. The free amino group content of this resin dropped from 0.07 to 0.015 mmole per g of substituted resin. Electrophoresis of the cleavage product showed one major and two minor spots of which one was identified as HCO-Tyr-N_2H_3 (XII).

HCO-Tyr-N_2H_3 (XII) Hydrazine hydrate (0.1 ml) was added to a solution of 223 mg of HCO-Tyr-OMe (1 mmole) in 15 ml of methanol. A precipitate formed after 2 days at 4°C. It was collected, washed with methanol and dried, yielding 189 mg (85%) of hydrazide XII, mp 218-220°C, $[\alpha]_D^{22}$ +16.7° (c 1, DMF), homogeneous (system B).

Anal. Calcd for $C_{10}H_{13}N_3O_3$: C, 53.81; H, 5.87; N, 18.82. Found: C, 53.8; H, 5.9; N, 18.5.

Reaction of Bmv-Gly-OH with the hydrazide resin (XIV,XVII) *p*-Toluenesulfonic acid monohydrate was dehydrated *in vacuo* (0.01 Torr) at 70°C and dissolved in dry methylene chloride. The solution was added to one equivalent of Bmv-Gly-OH.DCHA dissolved in the same solvent. This solution was added to the hydrazide resin VIb (0.43 mmole/g of resin). Coupling schedule A was applied. Deprotection of a sample was followed by free amino group determination, showing 0.22 mmole of free amino

groups/g of resin. A repeated coupling raised this value to 0.23. The deprotected sample was treated with 50% TFA in methylene chloride and chromatographed (system C). Besides a spot with R_F-value of 0 (H-Gly-N_2H_3), a spot with R_F-value of 0.7 was found which was U.V. and Reindel Hoppe positive, but Barton and ninhydrin negative. It had the same R_F-value as 5-methyl-3-phenyl pyrazole, obtained from benzoyl-acetone and $N_2H_4.H_2O$.

Azide coupling with amine bounded resin XVII (XVIII) 8 g of deprotected Bmv-Gly-hydrazide resin XVII were washed with DMAC (2 times, 2 minutes), suspended in 20 ml of the same solvent and left at -15°C. A solution of Z-Trp-Leu-N_2H_3[28] (3.72 g, 8 mmole) in 35 ml of DMF was cooled to -20°C and treated with 12.2 ml of 1.98N HCl/ethyl acetate (24 mmole), and then with 1.1 ml of *t*-butyl nitrite. After 20 min at -20°C the solution was cooled to -30°C and 3.36 ml of Et_3N (24 mmole) were added. This mixture was added to the resin suspension and rotated at 4°C. Samples were taken after one, six and eighteen hours. Free amino group determination gave 0.003 mmole/g of resin for the one hour sample. Identical values were obtained for the 6 and 18 hr samples. The excess of azide was removed from the resin by filtration and set apart. The resin was washed with DMF, methylene chloride, ethanol and methylene chloride, and treated with 30 ml of 50% TFA in methylene chloride for 30 min. Analysis of the products obtained by tlc showed two spots, one (R_F -value of 0.55, system C) was identical to the reference tripeptide hydrazide, the other (R_F-value of 0.7) was identical to 3-methyl-5-phenyl pyrazole.

Z-Trp-Leu-Gly-OEt The filtrate of XVIII, containing the excess of Z-Trp-Leu-N_3, was added to a cooled solution of 697 mg of HCl.H-Gly-OEt (5 mmole) and 0.7 ml of Et_3N (5 mmole). After 3 days at 4°C, the mixture was poured into water and extracted with ethyl acetate. The collected extracts were washed with bicarbonate solution, water, citric acid solution and water, and dried. Evaporation to a small volume and addition of petroleum ether gave a precipitate, which was collected and

dried, yielding 1.42 g (53%) of the protected tripeptide ethyl ester, mp 172-174°C, $[\alpha]_D^{22}$ -26.9° (c 1, MeOH), homogeneous (system A).

Anal. Calcd for $C_{29}H_{36}N_4O_6$: C, 64.91; H, 6.76; N, 10.44. Found: C, 64.75; H, 6.9; N, 10.4.

Z-Trp-Leu-Gly-N_2H_3 The tripeptide ethyl ester (0.6 g, 1.14 mmole) was dissolved in 15 ml of methanol, and 0.25 ml of hydrazine hydrate were added. After 2 days water was added and the precipitate collected, then recrystallized from ethanol yielding 0.55 g (93%) of the hydrazide, mp 186.5-187.5°C, $[\alpha]_D^{23}$ -26.8° (c 1, DMF), homogeneous (system C).

Anal. Calcd for $C_{27}H_{34}N_6O_5$: C, 62.05; H, 6.56; N, 16.08. Found: C, 62.0; H, 6.5; N, 16.1.

HCO-Cys(Trt)-Asn-OTmb (XXIV) A solution of 79 mg of HCl.H-Cys(Trt)-Asn-OTmb (XXIII, 0.122 mmole) in 2 ml of DMF was treated with 0.017 ml of Et_3N (0.122 mmole) and 0.5 ml of a 50% solution of isopropenyl formate in acetone. After 2 hr the suspension was diluted with 10 ml of water and extracted with ethyl acetate. The combined extracts were washed with water and dried over Na_2SO_4. The solution was concentrated to a small volume and petroleum ether was added. The precipitate was collected, yielding 50 mg (64%) of the formyldipeptide, amorphous, homogeneous (system A).

Anal. Calcd for $C_{37}H_{40}N_3O_5S$: C, 69.57; H, 6.31; N, 6.58. Found: C, 68.9; H, 6.0; N, 6.4.

Cyoc-ONp (XXV) A solution of 8 g of *p*-nitrophenyl chloroformate[29] in 40 ml of methylene chloride was added dropwise with stirring and cooling (0°C) to a solution of 3.9 g of 1-cyano-2-methyl-2-propanol[30] (39.8 mmole) in 40 ml of methylene chloride and 3.9 ml of pyridine. Stirring was continued at 0-4°C for 16 hr. The clear solution was poured into ice water and extracted with methylene chloride. The combined extracts were washed with 1N HCl solution and water, and dried. Evaporation of the solvent and recrystallizations from 2-propanol and ether-petroleum ether yielded 7.3 g (70%) of the carbonate, mp 75-78°C.

Anal. Calcd for $C_{12}H_{12}N_2O_5$: C, 54.55; H, 4.58; N, 10.60. Found: C, 54.4; H, 4.4; N, 10.5.

Cyoc-ONSu (XXVI) A solution of 2.75 g of cyano-*t*-butyl chloroformate[18] (17 mmole) in 10 ml of THF was added to a solution of 2.0 g of N-hydroxysuccinimide and 2.38 ml of Et_3N (17 mmole) in 10 ml of DMF. After 5 minutes the mixture was poured into water and extracted with ethyl acetate. The extract was washed with 0.1N HCl solution and water, and dried. Crystallization was effected from ethyl acetate-ether-petroleum ether, yielding 3.2 g (79%) of the carbonate, mp 93-94°C.

Anal. Calcd for $C_{10}H_{12}N_2O_5$: C, 50.00; H, 5.04; N, 11.66. Found: C, 50.0; H, 4.9; N, 11.5.

Msc-OPht(XXVII) A solution of 2.44 g of Msc-Cl[20] (13.1 mmole) in 5 ml of acetone was added dropwise to a cooled solution (0°C) of 2.14 g of N-hydroxyphtalimide (13.1 mmole) and 1.83 ml of Et_3N (13.1 mmole) in 10 ml of acetone. The addition of the Msc-Cl solution was terminated as soon as the red colour of the phtalimide ion disappeared. Water was then added and the precipitate collected and washed with water, yielding 3.84 g (93%) of the carbonate, mp 160-163°C.

Anal. Calcd for $C_{12}H_{11}NO_7S$: C, 46.01; H, 3.54; N, 4.47. Found: C, 46.2; H, 3.6; N, 4.4.

Msc-Cys(Trt)-Asn-OTmb (XXVIII) 194 mg of HCl.H-Cys(Trt)-Asn-OTmb (XXXIII, 0.3 mmole) and 60 mg of Msc-N_3[20] (0.31 mmole) were dissolved in 1 ml of methanol. Et_3N (0.042 ml, 0.3 mmole) was added and the solution left for 2 days at 20°C. Another equivalent of Msc-N_3 (0.3 mmole) as well as 0.021 ml of Et_3N (0.15 mmole) were then added. A crystalline precipitate, formed during the following 48 hr, was collected, washed with ethanol and water, and dried, yielding 158 mg (69%) of the protected dipeptide, mp 174-177°C, $[\alpha]_D^{23}$ +8.5° (c 1, DMF), homogeneous (system A,B).

Anal. Calcd for $C_{40}H_{46}N_3O_8S_2$: C, 63.14; H, 6.09; N, 5.52. Found: C, 62.85; H, 5.9; N, 5.4.

A_{14-19} hexapeptide bounded resins (XXX-XXXIIIb) The general schedules given were applied. Approximately 1 g of hydrazide resin was used. The N-terminal Z- and Msc amino protective groups were introduced by means of their phtalimido carbonates in DMF/CH_2Cl_2 as a solvent, using a tenfold excess. The reaction time was 16 hr. For the Bpoc cleavage and neutralization of the glutamine residue in resin XXXIIIb a shortened program was used: step 7 through 15 in schedule B were omitted. The coupling of Msc-Tyr(Bu^t)-OH[20] was DCC mediated with a reaction time of 90 minutes .

Msc-Tyr-Gln-Leu-Glu-Asn-Tyr-N_2H_3 The peptides were cleaved from the weighed resins (approximately 1.2 g) with 25 ml of 50% TFA in methylene chloride for 30 minutes at 20°C. The remaining resins were washed twice with 5 ml of methylene chloride. An I.R. spectrum of the resins obtained showed the almost complete absence of carbonyl absorption, indicating a nearly quantitative cleavage of the peptide. The filtrates combined for each sample were evaporated and each oily residue was dissolved in 12.5 ml of DMF. The addition of 50 ml of ethanol and 40 ml of ether caused a precipitate which was collected and dried over KOH. Tlc investigations of the purity of the obtained products were carried out in systems D and I. Only the hexapeptides obtained from resins XXXIIIa and XXXIIIb (automatized synthesis) were homogeneous. Their yields were 60-62%, based on the last Bpoc content measured. Mp 225-227°C, $[\alpha]_D^{23}$ -28.3° (c 1, DMF).

Anal. Calcd for $C_{42}H_{60}N_{10}O_{16}S$: C, 50.80; H, 6.09; N, 14.10. Found: C, 50.7; H, 6.0; N, 14.1.

Msc-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys(Trt)-Asn-OH (XXXIV) A solution of the hexapeptide hydrazide (obtained from XXXIIIa and XXXIIIb, 286 mg, 0.288 mmole) in 8 ml of DMF and 1 ml of DMSO was cooled to -20°C and treated with 0.105 ml of 7.5N HCl/DME (0.79 mmole) and 0.043 ml of *t*-butyl nitrite (0.375 mmole). The reaction mixture was stirred at -15°C for 20 minutes, then cooled to -30°C and 0.11 ml of Et_3N (0.79 mmole) were added. The solution of the azide was treated with a cooled solution of the dipeptide XL (167 mg, 0.35 mmole) in 3 ml of DMF. The pH of the solution was adjusted to 7 by means of a 10% Et_3N/DMF solution and left at 4°C for 3 days. The reaction mixture was diluted with 200 ml of

1-butanol-methanol-water (1:1:1) and added to an AG 1-X2 column (1.6x22 cm). The column was eluted subsequently with 100 ml of the same solvent, 1-butanol-methanol-0.3% acetic acid (300 ml), 1-butanol-methanol-1% acetic acid (100 ml), 1-butanol-methanol-3% acetic acid (200 ml) and, finally, 1-butanol-methanol-15% acetic acid (400 ml). Fractions of 5 ml each were collected. From absorbancy measurements at 254 nm and tlc, it was concluded that the 0.3% acetic acid eluate contained the excess of dipeptide XL, while the desired octapeptide was located in the 15% acetic acid eluate. Tubes containing chromatographically pure material were collected and evaporated. The residue was dissolved in 5 ml of DMF and precipitated with methanol/water, yielding 242 mg (58%) of the N,S-protected octapeptide. Mp 253°C (dec.), $[\alpha]_D^{23}$ -20.2°C (c 1, DMF), homogeneous (system I,E).

Anal. Calcd for $C_{68}H_{83}N_{11}O_{20}S_2.H_2O$: C, 56.07; H, 5.88; N, 10.58. Found: C, 55.9; H, 5.8; N, 10.5.

Amino acid ratios in acid hydrolysate (in the presence of phenol): Asp, 1.9; Glu, 2.0; Leu, 1.1; Tyr, 1.95.

H-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys(Trt)-Asn-OH (XXXV) A suspension of 105 mg of the above N,S-protected octapeptide (XXXIV, 0.073 mmole) was treated with 0.35 ml of 4N NaOH (0.14 mmole). After 5 minutes the clear solution was acidified with a few drops of 2N $KHSO_4$ solution and water was added. The precipitate was collected, washed with water and dried, yielding 85 mg (90.5%) of the S-protected octapeptide. Mp about 300°C (dec.) $[\alpha]_D^{23}$ -15.0° (c 1, DMF), homogeneous (system I).

Anal. Calcd for $C_{64}H_{77}N_{11}O_{16}S.2H_2O$: C, 58.04; H, 6.16; N, 11.63. Found: C, 57.9; H, 5.9; N, 11.5.

Amino acid analysis of a performic acid oxidized, acid hydrolysate: Asp, 2.0; Cys(O_3H), 1.0; Glu, 1.9; Leu, 1.0; Tyr, 1.5.

HCl.H-Asn-OPmb This compound was prepared according to Stewart's method for the preparation of the Tmb ester[31]. An overall yield of 66% was obtained. Recrystallization was effected from methanol-ethyl acetate. Mp 196-199°C, $[\alpha]_D^{23}$ -0.2° (c 0.5, methanol), homogeneous (system B).

Anal. Calcd for $C_{16}H_{25}N_2O_3Cl$: C, 58.44; H, 7.66; N, 8.52; Cl, 10.78.

Found: C, 58.3; H, 7.5; N, 8.5; Cl, 10.7.

Nps-Cys(Trt)-Asn-OPmb (XXXVII) A suspension of 3.1 g of Nps-Cys(Trt)-OH[32] (6 mmole) and 1.95 g of HCl.H-Asn-OPmb (6 mmole) in 50 ml of DMF was cooled to -10°C. This suspension was treated with 0.66 ml of NMM (6 mmole) followed by 0.69 g of N-hydroxysuccinimide (6 mmole) and 1.24 g of DCC (6 mmole). Stirring was continued for 2 hr at -10°C, 2 hr at 0°C and 16 hr at 20°C. The suspension was filtered, the filtrate poured into water and the precipitate collected and washed with bicarbonate solution, water, citric acid solution and water, and dried. Reprecipitation was effected by the addition of petroleum ether to a hot ethyl acetate solution, yielding 3.37 g (71%) of the protected dipeptide, mp 105-134°C, $[\alpha]_D^{23}$ -21.7° (c 1, MeOH), homogeneous (system F).

Anal. Calcd for $C_{44}H_{46}N_4O_6S_2$: C, 66.8; H, 5.86; N, 7.08; S, 8.11. Found: C, 66.7; H, 5.7; N, 7.1; S, 8.3.

Nps-Cys(Trt)-Asn-OTmb (XXXVI) The same procedure was applied as for the synthesis of the corresponding Pmb ester; HOBt was used instead of HONSu. A final yield of 98% was obtained, $[\alpha]_D^{24}$ -18.9° (c 1, MeOH), homogeneous (system F,C).

Anal. Calcd for $C_{42}H_{42}N_4O_6S_2$: C, 66.53; H, 5.81; N, 7.06. Found: C, 66.1; H, 5.6; N, 7.3.

HCl.H-Cys(Trt)-Asn-OTmb (XXIII) Compound XXXVI (3.81 g, 5 mmole) was dissolved in 10 ml of ethyl acetate and treated with 1.75 ml of 2-mercaptoethanol (25 mmole) and a mixture of 3.17 ml of 1.64N HCl-ethyl acetate (5.2 mmole) and 70 ml of ether. Petroleum ether was added after 5 min and the precipitate was collected. Reprecipitation was effected from ether-diisopropyl ether-petroleum ether, yielding 2.21 g (69%) of the salt XXIII, $[\alpha]_D^{22}$ +10.3° (c 1, DMF). It contained a trace of an impurity (system B,C).

Anal. Calcd for $C_{36}H_{41}N_3O_4SCl$: C, 66.80; H, 6.39; N, 6.49. Found: C, 66.2; H, 6.2; N, 6.5.

Boc-Cys(Trt)-Asn-OH (XXXIX) DCC (2.45 g, 11.8 mmole) was added to a stirred solution of S-trityl cysteine[33] (5.45 g, 11.8 mmole) and N-hydroxysuccinimide (1.36 g, 11.8 mmole) in 15 ml DME at -10°C. The reaction mixture was stirred at 0°C for 2 hr and at 20°C for 1 hr. The N,N-dicyclohexylurea formed was filtered and washed with DME (two 7.5 ml portions). The combined filtrates were added to a solution of 1.74 g of asparagine hydrate (11.8 mmole) and 1.63 g of K_2CO_3 (11.8 mmole) in 20 ml of water. DME (20 ml) was added to obtain a clear solution. After 90 min DME was evaporated and the residue acidified with a $KHSO_4$ solution. The resulting oil was extracted in ethyl acetate-ether (2:1), washed with water, and extracted again with 10% NMM solution and water. The combined extracts were acidified with 2N H_2SO_4 solution and extracted with ethyl acetate. The combined organic extracts were washed with water until neutral, dried and evaporated. A foam was obtained which was solidified with petroleum ether, yielding 5.97 g (88%) of the N,S-protected dipeptide, contaminated with small impurities (system C,G).
0.9 g of this material was dissolved in 100 ml of 1-butanol-methanol-water (1:1:1) and applied to an AG 1-X2 column (12x1.5 cm), which was eluted with the same solvent mixture followed by 1-butanol-methanol-1.5% acetic acid. Fractions containing homogeneous material were collected and evaporated. The residue was triturated with petroleum ether yielding 660 mg of pure XXXIX. $[\alpha]_D^{22}$ +29.0° (c 1, MeOH).

Anal. Calcd for $C_{31}H_{35}N_3O_6S$: C, 64.45; H, 6.11; N, 7.27. Found: C, 64.4; H, 5.9; N, 7.2.

H-Cys(Trt)-Asn-OH (XL) $BF_3.OEt_2$ (0.26 ml, 1.8 mmole) was added to a stirred solution of 355 mg of Boc-Cys(Trt)-Asn-OH (XXXIX, 0.615 mmole) in 5 ml of acetic acid. After 30 min the solution was poured into a sodium acetate solution. The resulting oil was extracted in 1-butanol (saturated with water) and the combined extracts were washed with water (saturated with 1-butanol) and evaporated. The oily residue was triturated with ether to yield 285 mg (97.5%) of S-protected dipeptide XL, $[\alpha]_D^{23}$ +27.7° (c 1, DMF), homogeneous (system B).

Anal. Calcd for $C_{26}H_{27}N_3O_4S.0.5H_2O$: C, 64.18; H, 5.80; N, 8.64. Found: C, 63.9; H, 5.6; N, 8.55.

Bpoc-Tyr(Bu^t)-Gln-OH (XLI) Bpoc-Tyr(Bu^t)-ONSu was prepared in 89% yield, mp 139-141°C (litt.[21] 66% yield, mp 136-138°C). The active ester (2.9 g, 5 mmole) was dissolved in 15 ml of DME and added to a solution of 0.73 g of glutamine (5 mmole) and 0.84 g of sodium bicarbonate (10 mmole) in 15 ml of water. To obtain a clear solution, 10 ml of DME was added. After 4 hr the DME was evaporated and at 0°C 10% citric acid solution was added. The resulting solid was extracted in ethyl acetate. The extract was washed with water, dried and concentrated to a small volume. Addition of ether and diisopropyl ether provided a precipitate of the desired dipeptide XLI: 2.34 g (78%), mp 132-134°C, $[\alpha]_D^{23}$ +13.5° (c 1, MeOH), homogeneous (system H).

Anal. Calcd for $C_{34}H_{41}N_3O_7 \cdot 0.25H_2O$: C, 67.14; H, 6.88; N, 6.91. Found: C, 67.2; H, 7.0; N, 6.85.

Bmv-Cys(Bzh)-OH (XLII) S-Benzhydryl cysteine[33] (5.49 g, 19.1 mmole) was suspended in 5 ml of water. Next, 3.77 ml of DCHA (19.2 mmole) dissolved in 16 ml of methanol was added, followed by the addition of 3.1 g of benzoylacetone (19.15 mmole) dissolved in 16 ml of ethanol. The mixture was refluxed with stirring for 3 hr. A clear solution was obtained. It was concentrated *in vacuo* and the residue dissolved in 2-propanol and evaporated again to dryness. This procedure was repeated once more. The residue was dissolved in ethyl acetate and shaken with a 10% citric acid solution at 0°C. The precipitate which formed was collected and washed with water and methanol, yielding 7.2 g (88%) of the free acid XLII, mp 185-187°C, $[\alpha]_D^{23}$ -195° (c 1, DMF), homogeneous (system H).

Anal. Calcd for $C_{26}H_{25}NSO_3$: C, 72.36; H, 5.84; N, 3.25. Found: C, 72.1; H, 5.5; N, 3.2.

The DCHA salt, prepared from the free acid, could only be obtained as an oil.

The CHA salt was precipitated from 2-propanol-ether, mp 130-137°C; $[\alpha]_D^{22}$ -111° (c 1, ethanol).

Anal. Calcd for $C_{32}H_{39}N_2O_3S$: N, 5.27. Found: N, 5.3.

Z-OPht (XLIV) N-Hydroxyphtalimide (114 g, 0.7 mole) was dissolved in 350 ml of DMF. The cooled (0°C) and stirred solution was treated with 98 ml of Et_3N (0.7 mole) and then with a 50% solution of phenyl chloroformate in toluene until the red colour of the phtalimide ion had disappeared (approximately 250 ml). The solution was filtered, the filtrate evaporated to a smaller volume and the resulting DMF solution poured into water. The precipitate was collected, washed with water and recrystallized from ethanol, yielding 176 g (85%) of the carbonate, mp 99-101°C.

Anal. Calcd for $C_{16}H_{11}NO_5$: C, 64.65; H, 3.73; N, 4.71. Found: C, 64.5; H, 3.7; N, 4.7.

Z-Tyr-OBzl (XLV) TOSOH.H-Tyr-OBzl (XLIII) was prepared in 74% yield, mp 179-181°C, according to Zervas *et al*[34]. The tosylate salt (133 g, 0.3 mole) was dissolved in 300 ml of DMF and treated with 44 ml of Et_3N (0.315 mole) and 94 g of Z-OPht (0.315 mole). The solution was left for 16 hr at 20°C and poured into 1500 ml of a 5% sodium bicarbonate solution. The resulting oil was extracted in ethyl acetate and washed with bicarbonate solution, water, 0.1N HCl solution and water, and dried. Evaporation of the solvent, followed by a recrystallization from methanol-ether-petroleum ether provided 112 g (92%) of XLV, mp 119-121°C, $[\alpha]_D^{22}$ -13.9° (c 1, MeOH), homogeneous (system F).

Anal. Calcd for $C_{24}H_{23}NO_5$: C, 71.1; H, 5.7; N, 3,45. Found: C, 71.3; H, 5.8; N, 3.4.

Z-Tyr(Bu^t)-OBzl (XLVI) 61.0 g of Z-Tyr-OBzl (0.143 mole) was dissolved in 140 ml of methylene chloride. The solution was treated in a pressure flask at -15°C with 1.5 ml of concentrated H_2SO_4 and 150 ml of isobutene. After 30 minutes a precipitate was observed, which dissolved after 5 hr. After 65 hr at 20°C the solution was poured into a 5% bicarbonate solution and nitrogen was bubbled through the solution for 15 min. The solvent was removed under reduced pressure and the residual oil extracted in ethyl acetate. The collected extracts were washed with water, cooled to 0°C and extracted twice with 2N NaOH at 0°C and once with NaH_2PO_4 solution. After final washings with water and brine, the solution was dried and evaporated. Crystallization from ethyl acetate-ether-petroleum

ether yielded 56 g (85%) of XLVI, mp 65-71°C. After one recrystallization 71-72.5°C, $[\alpha]_D^{25}$ -13.7° (c 1, MeOH), homogeneous (system F).

Anal. Calcd for $C_{28}H_{31}NO_5$: C, 72.86; H, 6.77; N, 3.03. Found: C, 72.5; H, 6.7; N, 3.1.

H-Tyr(Bu^t)-OH (XLVII) A solution of 11.52 g of compound XLVI (25 mmole) in 115 ml of methanol, 15 ml of water and 3.6 ml of acetic acid was hydrogenated in the presence of 5% palladium charcoal catalyst. The catalyst was removed by filtration and the filtrate was concentrated to a small volume. The precipitate was collected and dried. A second crop of H-Tyr(Bu^t)-OH was obtained from the filtrate, providing a total yield of 5.35 g (89%) of XLVII, mp 194-196°C, $[\alpha]_D^{23}$ -23.1° (c 1, H_2O, homogeneous (system D), lit.[25] mp 248-249.5°C, $[\alpha]_D^{23}$ -25.77° (c 1, H_2O).

Anal. Calcd for $C_{13}H_{19}NO_3 \cdot 0.25H_2O$: C, 64.57; H, 8.13; N, 5.79. Found: C, 64.6; H, 7.8; N, 5.8.

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C H A P T E R IV

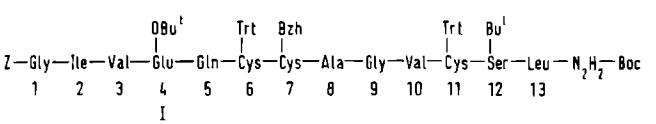
SYNTHESIS OF THE A_{1-13} FRAGMENT OF OVINE INSULIN

IV.1 INTRODUCTION

The strategy for the synthesis of the A_{1-13} segment of ovine insulin was determined by three requirements:

a. Selective protection of the thiol groups occurring in positions 6 and 11, compared with that in position 7, thus permitting the ultimate formation of the intrachain sulfur-sulfur bond between A_6 and A_{11}.
b. The protection of all side-chain functional groups with acid labile *tert*-butyl esters or ethers.
c. A C-terminal hydrazide function permitting comparison of intermediate products with solid-phase peptide derivatives, obtained from the Merrifield hydrazide resin.

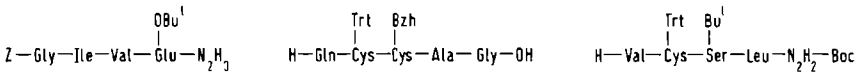
On that account the synthetic goal was the fully protected tridecapeptide derivative I:



Insulin fragments containing the S-trityl and S-diphenylmethyl protective groups have already been described by Zervas *et al*[1], and more recently by Hiskey *et al*[2]. The report of the latter describes also the use of thiocyanogen for the cyclization re-

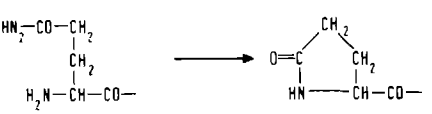
action.

The tridecapeptide was built up from three sub-units:



The final coupling was chosen after the glycine residue in position 9. An optically pure product can then be expected in the condensation of fragments 1-9 and 10-13.
Fragment 1-9 was synthesized from two smaller sub-units corresponding to the sequences 1-4 and 5-9.

A disadvantage in the condensation of fragments 1-4 and 5-9 might be cyclization of the N-terminal glutamine residue into the pyroglutamyl derivative:

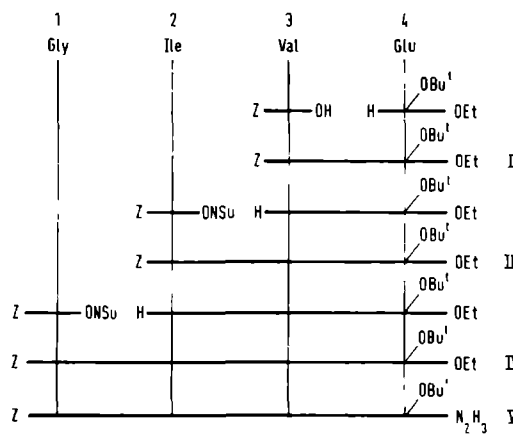


This side reaction can explain the relatively low yields, reported earlier in the coupling of A_{1-4} with A_{5-9}[3,4,5]. Therefore the Chinese group[6] applied a coupling between the A_{1-5} and A_{6-9} fragments in later investigations. They used glutamine-*tert*-butyloxycarbonyl hydrazide as a carboxyl protected glutamine derivative.

In the azide coupling between A_{1-4} and A_{5-9} however we obtained satisfactory yields of the desired nonapeptide. It could easily be separated from the contaminating pyroglutamyl peptide.

IV.2 THE SYNTHESIS OF THE A_{1-4} FRAGMENT

The synthesis of this fragment (V) has been described by Zahn[3] in his synthesis of the complete A chain. It was prepared by stepwise elongation of the peptide chain, starting from H-Glu(OBu^t)-OMe and using active ester couplings. In view of the fact that the parent ethyl ester is accessible more easily, we started our synthesis with H-Glu(OBu^t)-OEt, using the benzyloxycarbonyl group for the amino protection (scheme I).



Scheme I

The further synthesis of this tetrapeptide followed a known route. Mass spectral data were frequently used during the synthesis of this fragment and the A_{10-13} tetrapeptide, to identify side products, present in the unpurified peptides.

A methanolic solution of Z-Glu(OBu^t)-OEt was hydrogenated over palladium and the resulting amino ester was treated with

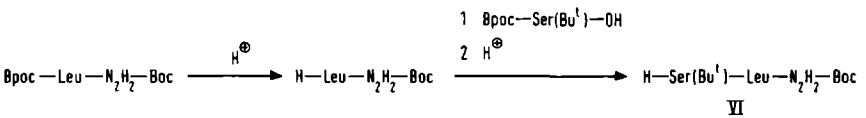
Z-Val-OH and DCC giving the dipeptide Z-Val-Glu(OBu^t)-OEt (II) in 80% yield. A mass spectrum revealed the presence of some acylurea in the reaction mixture. Katsoyannis[7] prepared this dipeptide in 79% yield, using a *p*-nitrophenyl ester coupling. The protected tripeptide Z-Ile-Val-Glu(OBu^t)-OEt (III) was readily obtained in crystalline form and in 53% yield by coupling an equivalent amount of Z-Ile-ONSu with the amino ester, obtained upon catalytic hydrogenation of the protected dipeptide II. Zahn[3] showed that neutral hydrogenation of Z-Val-Glu(OBu^t)-OMe in ethanol leads to the formation of a diketopiperazine. He reported 60% yield for the coupling between Z-Ile-ONp and the amino ester, obtained upon acidic hydrogenation. Katsoyannis[7] however, hydrogenated compound II in ethanol and isolated the tripeptide in 92% yield.
Hydrogenation of the protected tripeptide III, followed by coupling with Z-Gly-ONSu provided the protected tetrapeptide IV in 73% yield. Treatment of this tetrapeptide ethyl ester with hydrazine hydrate gave the hydrazide (V) in 85% yield. Physical constants of this tetrapeptide hydrazide were similar to those reported by Zahn[3] for the same compound.

IV.3 THE SYNTHESIS OF THE A_{10-13} FRAGMENT

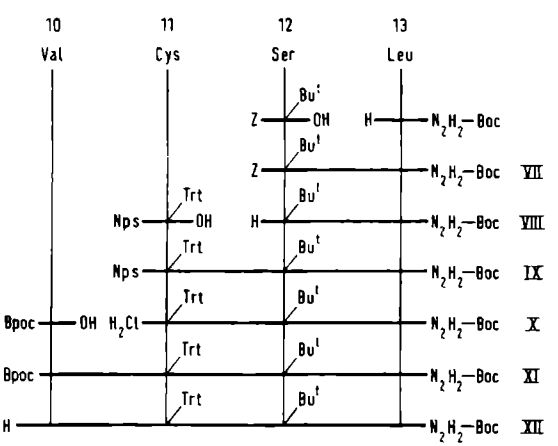
The presence of an S-trityl group and the required hydrazide-Boc protection in the C-terminus complicated the synthesis of this compound; *e.g.* in the final stages of the preparation the benzyloxycarbonyl group could no longer be used for amino protection. Although this protecting group could be applied for the synthesis of the C-terminal dipeptide, it seemed worth-while to investigate the use of the acid labile

Bpoc amino protection already at the early stages in the synthesis of this tetrapeptide.

It appeared however that the C-terminal dipeptide (VI) could not easily be obtained in a pure form in this manner. The oily products were contaminated with Bpoc cleavage products and could not easily be separated from these contaminations, due to comparable solubility properties.



The benzyloxycarbonyl group was used more successfully in the synthesis of this dipeptide. The Nps and Bpoc groups respectively, were chosen for the amino protection at the tri- and tetrapeptide level. The route finally used is shown in scheme 2.



Scheme 2

H-Leu-N_2H_2-Boc obtained by catalytic hydrogenation from the parent benzyloxycarbonyl compound was coupled with Z-Ser(Bu^t)-OH using DCC as condensing agent to give the completely protected dipeptide VII. Hydrogenation of this dipeptide was accomplished in methanol. The free amine was isolated in 70% yield.

The coupling reaction between Nps-Cys(Trt)-OH and the free aminopeptide VIII, using DCC/HOBt, proceeded smoothly and afforded the tripeptide derivative IX in 84% yield. Complete cleavage of the Nps group of IX was observed with exactly one equivalent of HCl in ethyl acetate and an excess of mercaptoethanol. The resulting hydrochloride salt of the amino peptide was soluble in ether; it was precipitated from the ethereal solution by the addition of petroleum ether, but some Nps-S-C_2H_4OH deposited also during this treatment. This raw material was used in the following coupling step without further purification.

The salt X was converted into the free base and coupled *via* the mixed anhydride method with Bpoc-Val-OH providing the protected tetrapeptide XI in 50% yield. An active ester coupling between Bpoc-Val-ONSu and the tripeptide X, resulted in even lower yields.

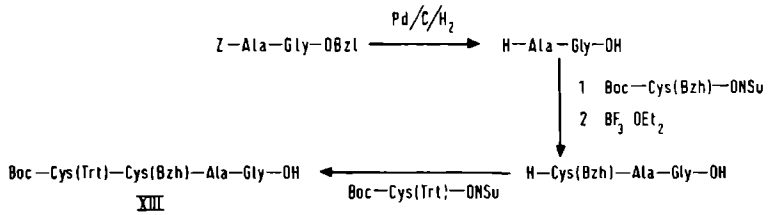
Treatment of XI with aqueous acetic acid at room temperature provided the free base in 93% yield. The product was homogeneous on tlc and gave the expected elemental analysis.

IV.4 THE A_{5-9} SEGMENT OF OVINE INSULIN

The synthesis of this fragment is more challenging than that of the two tetrapeptides described in the preceding sections. Complications can be expected because of the presence of a

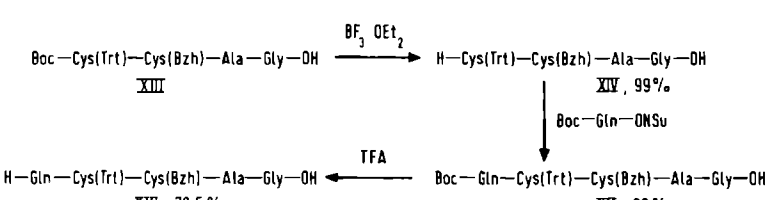
glutamine residue, the sulfur protective functions, and in consequence of the necessity to leave the C-terminal carboxyl group unprotected.

Recently Hiskey[2] described an elegant synthesis of the amino protected tetrapeptide A_{6-9}:

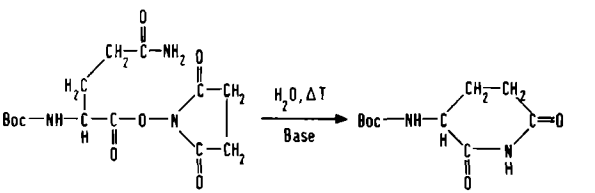


He applied two succinimide ester couplings with zwitterionic peptides. Boron trifluoride etherate was used to remove the Boc function[8]. We adopted this reaction scheme and succeeded in the synthesis of the protected tetrapeptide XIII within 2 weeks, starting from free amino acids. The overall yield, based on the tosylate salt of glycine benzyl ester, used as starting compound, was 40%. The tetrapeptide was homogeneous on tlc and had correct elemental analysis; a melting point of this peptide and of peptides derived from it, could not be determined. Upon heating these peptides turned black in a temperature range of 180-250°C without melting.

Cleavage of the Boc group from XIII was accomplished by treatment with boron trifluoride etherate in acetic acid:

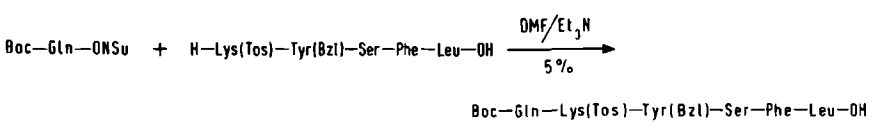


The next step *i.e.* the coupling of tetrapeptide XIV with Boc-Gln-ONSu can be accompanied by a side reaction which diminishes the yield of the desired pentapeptide XV. It is known [9,10,11] that succinimide esters of N-protected glutamine are unstable compounds, which cyclize easily to α-aminoglutarimides in water, at elevated temperatures, or under basic conditions.



Boc—Gln—ONSu Boc—L—α—aminoglutarimide

A striking example of this undesired reaction is found in a report of Li *et al* [12]; only 5% of the wanted hexapeptide was obtained in the following reaction:



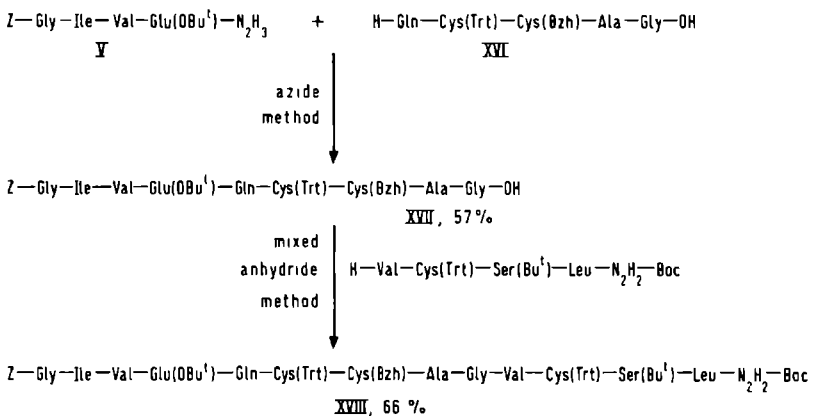
However we found that treatment of the tetrapeptide zwitterion XIV, dissolved in DMF, with a 25% excess of Boc-Gln-ONSu, without the addition of a base, resulted in the formation of the pentapeptide XV in 89% yield. Tlc showed that the conversion after 3 hours was approximately 50%; after 20 hours unreacted tetrapeptide XIV appeared to be completely absent.

As already cited in section IV.1, glutaminyl peptides without amino protecting groups can rearrange to pyroglutamyl derivatives. The appearance of this side product depends also on the acid used in the deprotection step XV → XVI. We found that treatment of Boc-Gln-OH with boron trifluoride etherate under standard conditions produced a trace of pyroglutamic acid as judged from tlc; the reaction from Boc-Gln-OH with trifluoroacetic acid however proceeded without any formation of pyroglutamic acid.
Because of these data, and the poor solubility of XV in acetic acid, the Boc function was cleaved with trifluoroacetic acid. The pentapeptide XVI was obtained in 78.5% yield. A 1% solution of this pentapeptide XVI, dissolved in DMF, was completely rearranged to the corresponding pyroglutamyl derivative within a week.

IV.5 THE COMPLETELY PROTECTED TRIDECAPEPTIDE A_{1-13}

Coupling of the three fragments, which have been described in the preceding sections was accomplished according to scheme 3.

The coupling of the tetrapeptide hydrazide V with freshly prepared pentapeptide XVI was carried out in DMF-DMSO. The resulting nonapeptide XVII, contaminated with some pyroglutamyl derivative could be purified by extracting the solid with boiling methanol. A final yield of 57% was obtained, which contrasts favourably with the 32% yield obtained by Zahn *et al* [3] in an azide coupling of V with a corresponding pentapeptide, containing S-benzyl protective groups.



Scheme 3

The solubility of XVII was rather poor. It dissolved in warm DMF, but from a 1% solution upon standing it separated as a gel. Thin layer chromatography was only possible with basic solvent systems.

The condensation between fragment XVII and XII to the tridecapeptide XVIII was realized with a mixed anhydride coupling. The nonapeptide XVII, which was dissolved in HMPA and to which some THF was added to prevent freezing of the solution, was treated with ethyl chloroformate. The tetrapeptide XII (10% excess) was dissolved in HMPA and the solution was added to the mixed anhydride. After one day a gelatineous precipitate had been formed and the fully protected tridecapeptide XVIII could be isolated in 71% yield. It had correct elemental analysis, and the amino acid analysis was in agreement with the composition expected. The observation that the peptide bond between residues A_2 and A_3 (-Ile-Val-) is cleaved very slowly during acid hydrolysis, has already been reported[3,13].

The bad solubility of the end-product XVIII prevented the establishment of its purity by tlc. DMF and DMSO or their mixtures did not appear to be useful to get the compound in solution. It dissolved very slowly in warm (60-70°C) HMPA but if the concentration exceeded 1% a precipitate was formed upon cooling to 20°C.

IV.6 REACTIONS WITH A_{1-13}

The extremely bad solubility of the fully protected tridecapeptide hydrazide XVIII, and the resulting lack of data concerning its purity, are serious drawbacks in its further use. Anyhow, products derived from the compound should have to be soluble to some extent in solvents like DMF, to permit purification at that stage. Prospects that this should be the case were rather unfavourable.

Because it has been our ultimate goal to prepare a derivative of the A chain, containing a disulfide bridge between the cysteine residues in positions A_6 and A_{11} and an S-benzhydryl function in position A_7, several attempts have been made to close the disulfide bridge in XVIII.

Hiskey[14] developed a method for ring closure of S-trityl protected peptides, using thiocyanogen, and demonstrated that S-benzhydryl functions are stable under these conditions. Prerequisite[15] to the application of this procedure is that the protected peptides are soluble in solvents like acetic acid or ethyl acetate because dimethylformamide or hexamethylphosphoric triamide are reactive towards thiocyanogen.
We treated a suspension of our protected tridecapeptide in acetic acid with thiocyanogen, but only the starting material

was recovered.

Kamber and Rittel[16] described the use of iodine for the synthesis of cystine peptides from the corresponding S-trityl protected cysteine peptides. They found that with one equivalent of iodine and a reaction time of one hour at 20°C S-benzhydryl groups in S-protected cysteine peptides are cleaved for 20%. The iodine cyclization method is therefore not fully specific but it can be carried out in polar solvent systems as DMF/methanol[17].
We investigated this technique for the preparation of cystine from S-trityl cysteine, using HMPA as a component of the solvent system, and found it quite suitable.

Exposure of the fully protected tridecapeptide, dissolved in HMPA/methanol (9:1) to one equivalent of iodine, dissolved in methanol, left a residue which was again insoluble in all conventional solvents. The presence of a disulfide bridge could not be established; a purification procedure was impossible.

When ten equivalents of iodine were used[16] a product soluble in DMF was formed. Thin layer chromatography revealed the presence of a major component with an R_F-value of 0.85 (n-BuOH-HOAc-H_2O = 4:1:1). Moreover 80% of the expected quantity of triphenylmethyl ether, the reaction product containing the tritylresidues, was obtained.

It appeared that during counter current distribution the soluble material transformed into very insoluble compounds; almost the whole sample precipitated in the first tubes of the apparatus.

We assume that the S-benzhydryl function was also cleaved to a large extent, producing a mixture of several disulfides, which are soluble because they also contain thiol groups. Upon

purification, the thiol residues are then oxidized to insoluble disulfides.

This hypothesis is supported by a mass spectrometrical investigation. As was expected, the mass spectra of tetrapeptide XIV as well as nonapeptide XVII, both containing one trityl and one benzhydryl function, showed peaks of the same intensity corresponding to the trityl and benzhydryl group. A mass spectrum of the iodine oxidized tridecapeptide also showed these peaks of the same intensity, corresponding to the benzhydryl and trityl groups. These must be due to residual traces of these groups, as 80% of the theoretical quantity of triphenylmethyl ether could be isolated.

This proves that both S-protecting groups have been cleaved unspecifically and rather completely, under the conditions used.

IV.7 CONCLUSION

The synthesis of the protected A_{1-4} (V) and A_{10-13} (XII) fragments of ovine insulin can be performed without complications. Difficulties, which might arise in the coupling and deprotection of the A_5 glutamine residue in an A_{5-9} segment can be overcome easily.

The coupling of the three fragments to the completely protected tridecapeptide A_{1-13} also proceeds in good yields. However, the product is only slightly soluble in hexamethylphosphoric triamide, due to its rather high molecular weight and especially low polarity. This prevents the use of this compound in the further preparation of the pure A chain.

Hiskey[2,18] has undertaken the synthesis of a tridecapeptide, containing less protective groups. He closed the disulfide

bridge in an A_{6-13} fragment, which was soluble in chloroform/ acetic acid. Coupling of the product with the A_{1-5} fragment provided, however, a tridecapeptide, which was also only soluble in HMPA/DMF mixtures. Thin layer chromatography failed, due to its insolubility.

We think that the C-terminal Boc-hydrazide group certainly contributes to the unfavourable solubility characteristics of the fragment. However, the main cause might be the presence of three very large apolar S-protecting groups (in our procedure) or of only one of them in combination with the disulfide bridge already formed (Hiskey's procedure).

It will be clear from the preceding part that the choice of the fragments upon which the thiocyanogen reaction is applied, is of the utmost importance. The main drawback of this method is its incompatibility with solvents like DMF or HMPA. Selective introduction of the disulfide bridges in insulin with the thiocyanogen method is therefore merely a matter of solubility.

IV.8 EXPERIMENTAL

General remarks given in sections II.9 and III.9 are also applicable to this section.

A list of solvent systems used for tlc is given in the appendix.

Mass spectra were measured with a double focussing mass spectrometer (Varian MATSM-1B)

Z-Val-Glu(OBu^t)-OEt (II) 10.95 g of Z-Glu(OBu^t)-OEt[19] (30 mmole) were dissolved in methanol and hydrogenated in the presence of 5% palladium charcoal catalyst. After complete disappearance of starting material had been demonstrated by tlc, the catalyst was filtered and the filtrate was concentrated to dryness. To a solution of the residue in 70 ml of DMF, 7.53 g of Z-Val-OH[20] (30 mmole) and 6.2 g of DCC (30 mmole) were added at -15°C. After 1 hr at -15°C and 16 hr at 0°C the N,N'-dicyclohexylurea which separated was filtered off. The filtrate was poured into 700 ml of water, and extracted with ethyl acetate. The extract was washed with sodium bicarbonate solution, water, citric acid solution and water, dried over sodium sulfate, and the solvent evaporated. The resulting oil was crystallized from ethyl acetate-petroleum ether, yielding 11.1 g (80%) of dipeptide II, mp 69.5-70.5°C (lit.[7] mp 70-71°C), homogeneous (system B).

Z-Ile-Val-Glu(OBu^t)-OEt (III) A solution of 7.9 g of compound II (17 mmole) in 100 ml of methanol and 1.5 ml of acetic acid was hydrogenated in the presence of 5% palladium charcoal catalyst. The catalyst was removed by filtration and the filtrate concentrated to dryness *in vacuo*. The residue was dissolved in 25 ml of DMAC and 6.15 g of Z-Ile-ONSu[21] (17 mmole) were added. After 18 hr at 0°C and 18 hr at 20°C the reaction mixture was poured into 500 ml of water and the precipitate was isolated by filtration. The product was dissolved in 250 ml of ethyl acetate at 40°C and washed with citric acid solution, water, sodium bicarbonate solution and water, and dried over sodium sulfate. The solution was reduced to 50 ml by evaporation. The tripeptide precipitated upon cooling,

providing 5.2 g (53%) of product III, mp 190-192°C (lit.[7] 186-189°C), homogeneous (system B).

Z-Gly-Ile-Val-Glu(OBu^t)-OEt (IV) A solution of 4.6 g of protected tripeptide III (8 mmole) in methanol was hydrogenated in the presence of 5% palladium charcoal catalyst. The catalyst was removed by filtration and the solvent was evaporated *in vacuo*. The residue was dissolved in 25 ml of DMAC and 2.5 g of Z-Gly-ONSu[21] (8 mmole) were added to the solution. After 12 hr at 4°C and 12 hr at 20°C, 700 ml of water were added under stirring. The precipitate which formed was collected and dried. Reprecipitation was effected from ethyl acetate-petroleum ether affording 3.71 g (73%) of tetrapeptide IV, mp 164.5-167°C, $[\alpha]_D^{22}$ -57.2° (c 2, methanol), homogeneous (system B).

Anal. Calcd for $C_{32}H_{50}N_4O_9$: C, 60.55; H, 7.94; N, 8.83. Found: C, 60.7; H, 8.1; N, 8.9.

Z-Gly-Ile-Val-Glu(OBu^t)-N_2H_3 (V) The tetrapeptide ethyl ester IV (3.71 g; 5.9 mmole) was dissolved in a mixture of 25 ml of methanol and 15 ml of DMF, and treated with 2.2 ml of hydrazine hydrate. The solution was being stirred at room temperature during 4 days and diluted with water. Reprecipitation was effected from methanol, yielding 3.11 g (85%) of tetrapeptide hydrazide V, mp 238-240°C, $[\alpha]_D^{22}$ -13.5° (c 1, DMF) lit.[3] mp 243-244°C, $[\alpha]_D^{25}$ -13.8° (c 1, DMF), homogeneous (system A).

Anal. Calcd for $C_{30}H_{48}N_6O_8$: C, 58.14; H, 7.64; N, 13.56. Found: C, 58.0; H, 7.8; N, 13.5.

Z-Ser(Bu^t)-Leu-N_2H_2-Boc (VII) A stirred solution of 5.9 g of Z-Ser(Bu^t)-OH[22] (20 mmole) and 4.9 g of H-Leu-N_2H_2-Boc[23] (20 mmole) in 25 ml of ethyl acetate and 25 ml of DMF was cooled to -15°C. 4.12 g of DCC (20 mmole) were then added to the solution. Stirring was continued for 16 hr at 0°C and for 2.5 hr at 20°C. The precipitated N,N'-dicyclohexylurea was collected by filtration, the filtrate poured into 200 ml of water and extracted with ethyl acetate. The organic extract was washed with citric acid solution, water, sodium bicarbonate solution and water, and dried over sodium sulfate. The solution was concentrated to a small volume *in*

vacuo. Addition of hexane gave a precipitate which was collected and dried to give 7.7 g (74%) of dipeptide VII, mp 152-154°C, $[\alpha]_D^{23}$ -23.0° (c 1, MeOH), homogeneous (system A).

Anal. Calcd for $C_{26}H_{42}N_4O_7$: C, 59.57; H, 8.10; N, 10.72. Found: C, 60.4; H, 8.1; N, 10.7.

H-Ser(Bu^t)-Leu-N$_2$H$_2$-Boc (VIII) A solution of compound VII (5.22 g, 10 mmole) in 100 ml of methanol was treated with 0.5 g of 5% palladium on charcoal and hydrogen was bubbled through for 1 hr. The catalyst was filtered and the filtrate evaporated, leaving a white solid. This was crystallized from ethyl acetate-hexane to yield 2.7 g (70%) of dipeptide VIII, mp 88-91°C, homogeneous (system A).

Anal. Calcd for $C_{18}H_{36}N_4O_6$: C, 55.65; H, 9.34; N, 14.42. Found: C, 55.7; H, 9.4; N, 14.25.

Nps-Cys(Trt)-Ser(Bu^t)-Leu-N$_2$H$_2$-Boc (IX) A stirred solution of dipeptide VIII (1.94 g, 5.0 mmole) and 2.58 g of Nps-Cys(Trt)-OH[24] (5.0 mmole) in 50 ml of DMF was cooled to -10°C and treated with 0.75 g of HOBt (5.0 mmole) and 1.03 g of DCC (5.0 mmole). After 16 hr at 4°C, another equivalent of HOBt was added. The mixture was stirred for 36 hr at 4°C and the precipitated N,N'-dicyclohexylurea was collected. The filtrate was poured into water and extracted with methylene chloride. The extract was washed with $KHSO_4$ solution, water, sodium bicarbonate solution and water, and dried over sodium sulfate. Concentration of the solution *in vacuo*, followed by trituration with petroleum ether provided 3.7 g (84%) of tripeptide IX, mp 126-128°C, homogeneous (system B).

Anal. Calcd for $C_{46}H_{58}N_6O_8S_2$: C, 62.28; H, 6.59; N, 9.47. Found: C, 62.6; H, 6.8; N, 9.3.

HCl.H-Cys(Trt)-Ser(Bu^t)-Leu-N$_2$H$_2$-Boc (X) A stirred solution of IX 3.73 g, 4.2 mmole) in 15 ml of methylene chloride was treated with 1.5 ml of mercaptoethanol (21 mmole) and a solution of 2.25 ml of 1.87N HCl in ethyl acetate (4.2 mmole), diluted with 60 ml of ether. After standing at room temperature for 5 minutes the solution was poured into 200 ml of ether and cooled to -10°C. Petroleum ether was added and the off-white

precipitate was collected to give 2.76 g (85%) of the impure product X, which was used without purification in the preparation of tetrapeptide XI.

Bpoc-Val-Cys(Trt)-Ser(Bu^t)-Leu-N_2H_2-Boc (XI) A solution of Bpoc-Val-OH[25] (0.66 g, 1.85 mmole) in 7.5 ml of DMF was treated at -15°C with 0.23 ml of pivaloyl chloride and 0.2 ml of N-methylmorpholine (1.85 mmole). The suspension was stirred for 15 minutes at -10°C. Then a solution of X (1.44 g, 1.96 mmole) and 0.2 ml of N-methylmorpholine in 5.6 ml of DMF was added. Stirring was continued for 16 hr at 0°C. The mixture was poured into 100 ml of water and extracted with ethyl acetate. The organic layer was washed with citric acid solution, water, sodium bicarbonate solution and water, and dried over sodium sulfate. Removal of the solvent by evaporation and crystallization of the residue from ether-hexane provided 0.98 g (50%) of tetrapeptide XI, mp 201-204°C, $[\alpha]_D^{22}$ -11.5° (c 1, chloroform), homogeneous (system A,B).

Anal. Calcd for $C_{61}H_{78}N_6O_9S.0.5H_2O$: C, 67.81; H, 7.37; N, 7.78. Found: C, 67.5; H, 7.1; N, 7.9.

H-Val-Cys(Trt)-Ser(Bu^t)-Leu-N_2H_2-Boc (XII) A suspension of XI (0.89 g, 0.82 mmole) in 30 ml of 80% acetic acid was stirred at room temperature for 16 hr. The clear solution was poured into 150 ml of ice water and sodium carbonate was added to adjust the pH to 7.5-8.0. The oil, which separated was extracted in ethyl acetate. The combined extracts were washed with water, dried over sodium sulfate and concentrated to dryness *in vacuo*. Solidification of the oily residue was effected from ethyl acetate-petroleum ether, yielding 0.62 g (93%) of tetrapeptide XII, mp 208-211°C, $[\alpha]_D^{22}$ -10.2° (c 0.6, MeOH), homogeneous (system A).

Anal. Calcd for $C_{45}H_{63}N_6O_7S$: C, 64.88; H, 7.74; N, 10.09. Found: C, 65.1; H, 8.0; N, 9.7.

H-Cys(Trt)-Cys(Bzh)-Ala-Gly-OH (XIV) A suspension of 5.52 g of Boc-Cys(Trt)-Cys(Bzh)-Ala-Gly-OH[2] (6.41 mmole) in 65 ml of acetic acid was stirred at 60°C until an almost clear solution had been obtained. The solution was cooled to 20°C, and 2.6 ml of boron trifluoride etherate

(18 mmole) were added. After 45 minutes the solution was filtered and poured into 20% aqueous sodium acetate solution (100 ml). Ice was added and the precipitate was collected and washed with water and ether, yielding 4.87 g (99%) of a white solid. $[\alpha]_D^{23}$ +2.02° (c 1.09, DMF), homogeneous (system J). A sample for analysis was precipitated from a solution in DMF by the addition of water.

Anal. Calcd for $C_{43}H_{44}N_4O_5S_2 \cdot 0.5H_2O$: C, 67.08; H, 5.89; N, 7.28. Found: C, 67.7; H, 5.9; N, 7.2.

Boc-Gln-Cys(Trt)-Cys(Bzh)-Ala-Gly-OH (XV) A suspension of 4.45 g of tetrapeptide XIV (5.85 mmole) in 100 ml of DMF was stirred at 45°C until a clear solution was obtained. After cooling to 20°C, 2.6 g of Boc-Gln-ONSu[26] (7.5 mmole) were added, and the solution was stirred for 20 hr. The solution became clear after 3 hr. After the addition of 50 ml of ethanol, 50 ml of ethyl acetate and 100 ml of ether a precipitate was formed. It was left overnight at 0°C, then collected by filtration and washed with ethanol and water, yielding 5.13 g (89%) of pentapeptide XV. $[\alpha]_D^{23}$ -17.2° (c 1.15, DMF), homogeneous (system E,J).

Anal. Calcd for $C_{53}H_{60}N_6O_9S_2 \cdot H_2O$: C, 63.20; H, 6.20; N, 8.34. Found: C, 63.3; H, 6.1; N, 8.6.

H-Gln-Cys(Trt)-Cys(Bzh)-Ala-Gly-OH (XVI) 989 mg of protected pentapeptide XV (1 mmole) was treated with 30 ml of trifluoroacetic acid. The clear yellow solution was left at 20°C for 30 minutes and evaporated *in vacuo*. The remaining yellow oil was triturated with dry ether, and the resulting white solid collected and dried for 30 minutes *in vacuo* over KOH. The solid was dissolved in 50 ml of DMF (under gentle warming), and a 10% Et_3N/DMF solution was added until the pH was neutral. The solution was poured into 60 ml of ethanol and the precipitate collected, yielding 690 mg (78.5%) of pentapeptide XVI. Homogeneous (system J,K). It was used directly for the synthesis of nonapeptide XVII.
In a 1% solution in DMF the free pentapeptide was completely converted into the corresponding pyroglutamyl peptide within one week.

Z-Gly-Ile-Val-Glu(OBu^t)-Gln-Cys(Trt)-Cys(Bzh)-Ala-Gly-OH (XVII) *tert*-Butyl nitrite (0.093 ml, 0.78 mmole) was added to a solution, cooled to -20°C, of tetrapeptide hydrazide V (372 mg, 0.6 mmole) in 7 ml of DMF containing 1.65 mmole of HCl in ethyl acetate. The mixture was stirred at -20°C for 30 minutes and cooled to -40°C. Then 0.231 ml of Et_3N (1.65 mmole) was added, followed by a cooled solution of pentapeptide XVI (445 mg, 0.5 mmole) in 7 ml of DMSO-DMF (1:3). The pH was adjusted to 7.5-8.0 by addition of 10% Et_3N/DMF solution. After 16 hr at 4°C the pH was adjusted again to 7.5-8.0. After another 3 hr at 4°C, tlc revealed the absence of starting products. The suspension was poured into methanol-acetic acid and the gelatinous precipitate collected, washed with methanol and water, and dried. The solid was treated with boiling methanol (2 times), collected and dried, yielding 422 mg (57%) of nonapeptide XVII. $[\alpha]_D^{23}$ +5.5° (c 1, HMPA), homogeneous (system J).

Anal. Calcd for $C_{78}H_{96}N_{10}O_{15}S_2.H_2O$: C, 62.63; H, 6.60; N, 9.36. Found: C, 62.25; H, 6.5; N, 9.4.

Z-Gly-Ile-Val-Glu(OBu^t)-Gln-Cys(Trt)-Cys(Bzh)-Ala-Gly-Val-Cys(Trt)-Ser(Bu^t)-Leu-N_2H_2-Boc (XVIII) Nonapeptide XVII was dried at 80°C. 251 mg (0.17 mmole) were dissolved at 50°C in 7.5 ml of HMPA and 3 ml of THF. The solution was cooled (-30°C) and treated with 0.025 ml of Et_3N (0.18 mmole) followed by 0.0164 ml of ethyl chloroformate (0.17 mmole) After standing at -20°C during 15 minutes a cold solution of 150 mg of tetrapeptide XII (0.18 mmole) in 2 ml of HMPA was added. After 24 hr at 0°C a gelatinous precipitate was formed. DMF was added and the suspension was stirred at 60-80°C for 10 minutes. The precipitate was collected and washed with DMF, water and methanol, and dried, yielding 277 mg (71%) of completely protected tridecapeptide, $[\alpha]_D^{23}$ -12.1° (c 0.52, HMPA).

Anal. Calcd for $C_{123}H_{158}N_{16}O_{21}S_3$: N, 9.77; S, 4.20. Found: N, 9.7; S, 4.4.

Amino acid analysis: Cys(O_3H) 2.5, Ser 0.8, Glu 2.0, Gly 2.0, Ala 1.2, Val 1.5, Ile 0.7, Leu 0.9.

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SAMENVATTING

Het in dit proefschrift beschreven onderzoek had tot doel meer inzicht te verwerven in de synthese van insulinefragmenten, onder gebruikmaking van zuurlabiele, selectief afsplitsbare zwavelbeschermgroepen aan de cysteïne resten.

Zowel de synthese in oplossing als de synthese van fragmenten met behulp van de vaste-fase techniek werden onderzocht.

Het gebruik van selectieve zwavelbescherming maakt het wellicht mogelijk te komen tot een totaalsynthese van insuline waarin ook de disulfidebruggen op een eenduidige manier zijn aangebracht. Voor deze disulfide vorming kan gebruik gemaakt worden van de door Hiskey ontwikkelde thiocyanogeen methode.

Hoofdstuk I geeft een kort overzicht van de tot op heden verrichte onderzoekingen naar de synthese van insuline. De resultaten van orienterend onderzoek over de selectieve invoering van disulfidebruggen met behulp van thiocyanogeen worden nader uiteengezet.

Hoofstuk II handelt over de eventuele toepasbaarheid van een nieuw ontwikkelde vaste drager (hydroxy-ethyl-sulfonylmethylhars, β-sulfon hars) voor de synthese van het C-eindstandige A_{14-21} fragment.
De koppeling van C-eindstandig asparagine met het β-sulfon hars werd uitvoerig onderzocht. Het bleek, dat in een S-acyl-2-mercapto ethylester van N-beschermd asparagine de O-ester eerder transesterificatie ondergaat dan de S-ester. Een indirecte invoering van asparagine via het chloormethyl hars, was daardoor niet mogelijk.

Een verestering met behulp van N,N-dimethylformamide-dineopentylacetaal leidde wel tot een goede incorporatie van asparagine in het β-sulfon hars. Afsplitsing van dit aminozuurresidu (onder β-eliminatie condities) gaf echter aanleiding tot de vorming van het overeenkomstige succinimide derivaat. Bescherming van de amidefunctie van asparagine onderdrukte deze succinimide vorming; de combinatie echter van de amidebeschermgroep met andere zuurlabiele groepen voor de tijdelijke bescherming van α-aminofuncties resulteerde reeds bij de verdere uitbouw tot een dipeptide-hars in inhomogene producten.

In hoofdstuk III is een uitvoerige beschrijving gegeven van methodologische onderzoekingen met Merrifield's tert-alkyloxycarbonylhydrazide hars. Een verbeterde synthese voor deze drager werd gevonden, alsmede een procedure om snel en eenvoudig Bpoc- en Bmv-aminozuur incorporaties te meten met behulp van kwantitatieve U.V. metingen.
Het grote belang van het blokkeren van vrijgebleven aminogroepen na opeenvolgende aminozuur koppelingen werd aangetoond. Er werd een reagens gevonden (isopropenyl formaat), dat hiervoor bijzonder geschikt bleek, omdat het onder neutrale omstandigheden specifiek aminofuncties formyleert.
De inbouw van cysteïnederivaten via diverse koppelingsmethodieken werd onderzocht; de incorporatie van deze derivaten bleek bij geen der beproefde methoden volledig genoeg om succesvol toegepast te kunnen worden voor de synthese van langere sequenties.
Het bleek dat de reeds bekende p-nitrofenylester koppeling voor asparagine en glutamine in de vaste-fase synthese met goed resultaat vervangen kan worden door een DCC/HOBt koppeling.

In hoofdstuk III wordt verder ingegaan op de invoering en afsplitsing van verschillende aminobeschermgroepen, die ten opzichte van trifluoroazijnzuur stabiel zijn. Afsplitsingsexperimenten toonden aan, dat de Msc-groep (via β-eliminatie af te splitsen) alleen in combinatie met niet veresterd asparagine gebruikt kan worden.

Deze methodologische onderzoekingen hebben geleid tot een goede procedure voor de synthese van het A_{14-19} fragment. Herhaald in een "peptide synthesizer" bleek de geautomatiseerde synthese duidelijk superieur aan de "hand" methode. Het peptide, dat onder gebruikmaking van DCC/HOBt koppelingen en de Msc groep voor N-eindstandige aminobescherming werd verkregen, was zuiver. Het werd in oplossing verder gekoppeld met het C-eindstandige, S-beschermde dipeptide van de A keten, uiteindelijk resulterend in het S-beschermde octapeptide A_{14-21}.
In de laatste fase van deze synthese werd het gebruik van een AG 1-X2 ionenwisselaar kolom voor zuiveringsprocedures als zeer waardevol bevonden.

Hoofdstuk IV beschrijft de synthese in oplossing van een volledig beschermd A_{1-13} fragment, waarin met thiocyanogeen specifiek een intramoleculaire disulfidebrug tussen A_6 en A_{11} aangebracht zou kunnen worden.
De problemen die optreden bij de koppeling en deblokkering van het glutamineresidu in positie A_5 werden tot een elegante oplossing gebracht, zodat het A_{1-9} fragment in een relatief hoge opbrengst kon worden verkregen.
Het volledig beschermde tridecapeptide bleek helaas uitzonderlijk moeilijk in oplossing te brengen. De intramoleculaire disulfidebrug vorming o.i.v. thiocyanogeen, noodzakelijker-

wijze uit te voeren in conventionele oplosmiddelen, was dan ook niet mogelijk. Ringsluiting o.i.v. jodium bleek onvoldoende selectief te zijn.
Bij het ontwikkelen van een goede strategie voor de synthese van insulinesequenties zal de oplosbaarheid van de intermediairen dan ook een bepalende factor zijn voor het welslagen van de thiocyanogeen methode.

APPENDIX

A. List of solvent systems used for tlc

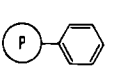
(A) chloroform-methanol (9:1)
(B) 1-butanol-acetic acid-water (4:1:1)
(C) chloroform-methanol (7.5:2.5)
(D) heptane-*tert*-butanol-acetic acid-water-pyridine (25:70:6:24:20)
(E) 1-butanol-acetic acid-water-pyridine (16:1:4:3)
(F) benzene-acetone (1:1)
(G) n-heptane-1-butanol-acetic acid (3:1:1)
(H) 1-butanol-acetic acid-water-pyridine (30:6:24:20)
(I) 1-butanol-acetic acid-water-pyridine (4:1:2:1)
(J) chloroform-methanol-17% ammonia (6:6:1)
(K) chloroform-methanol-17% ammonia (24:6:1)

B. Abbreviations

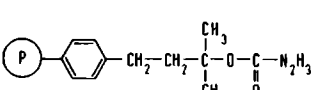
The abbreviations used are mainly those recommended by the IUPAC commission on biochemical nomenclature [Biochemistry 11, 1726 (1972)]

Bmv	2-benzoyl-1-methylvinyl
Boc	*t*-butyloxycarbonyl
Bpoc	2-(*p*-biphenyl)-isopropyloxycarbonyl
Bu^t	*t*-butyl
Bzh	benzhydryl (diphenylmethyl)
Bzl	benzyl

CHA	cyclohexylamine
Cyoc	cyano-*t*-butyloxycarbonyl
DCC	N,N'-dicyclohexylcarbodiimide
DCHA	dicyclohexylamine
DIEA	N-ethyldiisopropylamine
DMAC	dimethylacetamide
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DME	1,2-dimethoxyethane
Et	ethyl
HMPA	hexamethylphosphoric triamide
HOBt	N-hydroxybenzotriazole
Im	imidazole
Me	methyl
Msc	methylsulfonylethyloxycarbonyl
NMM	N-methylmorpholine
Nps	*o*-nitrophenylthio
ONb	*p*-nitrobenzyloxy
ONp	*p*-nitrophenoxy
ONSu	succinimido-oxy
OPht	phtalimido-oxy
OPmb	pentamethylbenzyloxy
OTmb	2,4,6-trimethylbenzyloxy
Py	pyridine



2% crosslinked polystyrene



(hydrazide resin)

s.p.p.s.	solid phase peptide synthesis
TFA	trifluoroacetic acid
THF	tetrahydrofurane
tlc	thin layer chromatography
TOSOH	*p*-toluenesulfonic acid
Trt	trityl (triphenylmethyl)
Z	benzyloxycarbonyl

Curriculum Vitae

Na het behalen van het diploma H.B.S.-B in 1962 aan het Ludgercollege te Doetinchem werd in datzelfde jaar begonnen met de studie in de scheikunde aan de Nijmeegse Universiteit. Het doctoraalexamen met als hoofdvak organische chemie en als bijvakken ekonomie, en fysische en chemische technologie, werd afgelegd in juli 1968. Gedurende het daarop volgende jaar werd aan de University of North Carolina deelgenomen aan een project betreffende de synthese van insuline onder leiding van Prof.dr. R.G. Hiskey. In oktober 1969 werd dit werk met het in dit proefschrift beschreven onderzoek voortgezet in het Organisch Chemisch Laboratorium van de Universiteit van Nijmegen onder leiding van Prof.dr. R.J.F. Nivard en Dr. G.I. Tesser.

STELLINGEN

I

De synthese van het epidithio-dioxo-piperazine ringsysteem via de niet overbrugde dioxo-piperazine ring kan niet model staan voor de synthese van de bekende natuurstoffen die dit bicyclische ringsysteem bevatten.

H. Poisel en U. Schmidt, Chem. Ber. **105**, 625 (1972)

II

Bij vele peptide synthetische methoden met vaste dragers wordt onvoldoende rekening gehouden met de mogelijkheid van carbaminezuurvorming.

III

De β-eliminatie van de methyl-sulfonyl-ethyloxycarbonyl groep verloopt waarschijnlijk volgens E1cB mechanisme.

F.G. Bordwell, Accounts Chem. Res. **5**, 374 (1972)
L.A. Carpino en C.Y. Han, J. Org. Chem. **37**, 3404 (1972)

IV

De negatieve resultaten verkregen door Wilchek met betrekking tot de affiniteitschromatografie aan c-AMP-sepharose kunnen verklaard worden uit een onvoldoend rendement in de eiwit-ligand binding, gekoppeld aan een ononderkende labiliteit van de verankerende imino-ether band.

M. Wilchek, Y. Salomon, M. Lowe en Z. Selinger,
Biochem. Biophys. Res. Commun. **44**, 305 (1971)
G.I. Tesser, H.U. Fisch en R. Schwyzer, FEBS Letters **23**, 56 (1972)

V

Voor een goed begrip van peptide-chemische verhandelingen is het kunnen denken in codes onontbeerlijk.

VI

Publikaties van vaste fase synthese van langere peptides dienen alleen geaccepteerd te worden, indien in de titel 'vermoedelijke synthese' of een synoniem daarvan voorkomt.

VII

Het is onmogelijk dat er een verschil bestaat tussen de mediaan-zuurstofdruk en de druk voor halfverzadiging van hemoglobine moleculen, waarin het heme-ijzer in de β-ketens in de Fe^{3+} vorm voorkomt.

R. Banerjee en R. Cassoly, J. Mol. Biol. 42, 351 (1969)

VIII

Bij de bestudering van het initiatiemechanisme van de eiwitsynthese in eukaryoten is het gebruik van initiator tRNA geïsoleerd uit prokaryoten onjuist.

R.P. McCroskey, M. Zasloff en S. Ochoa, Proc. Nat. Acad. Sci. USA 69, 2541 (1972)

IX

Bij de interpretatie van de onderlinge verschillen in reactiviteit van styreenderivaten in de reactie met het tosylradikaal wordt door Waters ten onrechte het para-effect buiten beschouwing gelaten.

C.M.M. da Silva Corrêa en W.A. Waters, J.C.S. Perkin II 1972, 1575
M. Sakurai, S.-i. Hayashi en A. Hosomi, Bull. Chem. Soc. Jap. 44, 1945 (1971)

X

Van alle argumenten die milieubeschermers aanvoeren tegen destructie van de groene vegetatie, is de bewering dat dit zal leiden tot een tekort aan atmospherische zuurstof slechts beperkt geldig.

XI

Scheikunde als natuurwetenschap heeft dringend behoefte aan een bezinning op haar opdracht als wetenschap van de natuur.

Nijmegen, 18 mei 1973 E.Th.M. Wolters